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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Serial No.: 09/980,062	)	
Filed: May 10, 2002	)	
Applicant: Naidu, Satyanarayan A.	)	
For: IMMOBILIZED LACTOFERRIN (Im-LF) ANTIMICROBIAL AGENTS AND USES THEREOF	)	Examiner: Russel, Jeffrey E. Group Art Unit: 1654
(US-PCT-106099)	)	

REPLACEMENT APPEAL BRIEF
Appeal of Final Office Action of April 7, 2005

CERTIFICATE OF MAILING (37 CFR 1.8(A))
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Sir:

Enclosed is a Replacement Appeal Brief.

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(1) Real Parties in Interest

A. Satyanarayan Naidu, an individual living at 9200 Monte Vista Ave., No. 3, Montclair, CA 91763, LF Tech, a limited liability company having an office at 299 S. Main Street, Suite 2450, Salt Lake City, UT 84111, and aLF Ventures, a limited liability company having an office at 299 S. Main Street, Suite 2450, Salt Lake City, UT 84111 are the real parties in interest.

(2) Related Appeals and Interferences

Neither Appellant, Appellant's legal representative, or his assignees are aware of any other appeals or interferences which will directly affect or be directly affected by or have a bearing on the Board's decision in the pending appeal.

(3) Status of Claims

Currently pending are claims 1-49, 51, and 56-202. Claims 6-10, 14-17, 40-49, 51, 59-61, 63, 66, 67, 69-85, 91, 93-100 and 118 have been allowed. Claims 3, 4, 12, 13, 21, 23-27, 29, 30, 33-37, 56-58, 62, 64, 65, 68, 87-90, 92, 105, 107-114, 125, 130, 139-141, 152, 155, 156, 160, 161, 166-170, 174, 177, 178, 182, 183, 188-192, 198, and 199 have been objected to. Claims 1, 2, 5, 11, 18-20, 22, 28, 31, 32, 38, 39, 86, 101-104, 106, 115-117, 119-124, 126-129, 131-138, 142-151, 153, 154, 157-159, 162-165, 171-173, 175, 176, 179-181, 184-187, 193-197, and 200-202 have been rejected.

(4) Status of Amendments

The Examiner mailed a Final Office Action on April 7, 2005. The Applicant filed a proposed amendment on August 26, 2005. In an Advisory Action mailed September 13, 2005, the Examiner notified the Applicant that for the purposes of this appeal, the proposed amendments would not be entered.

(5) Summary of Claimed Subject Matter

This application relates to antimicrobial agents and their use. (Specification, page 1, line 10.) Lactoferrin (LF) is a known antimicrobial agent. (Specification, page 8, lines 10 and 11.) LF is a glycol-protein having a bilboate structure, with an N-terminus lobe and a C-terminus lobe. (Specification, page 8, lines 15 and 16.) The activity of LF is highly dependent on its three-dimensional or tertiary structure. (Specification, page 8, lines 23 and 24.) If the protein dos not have the proper conformation, its activity is diminished or lost. (Specification, page 8, lines 24 and 25.) LF can be immobilized by mixing the LF with the naturally occurring substrate in a suitable medium, such as deionized water. (Specification, page 13, lines 5-7.) Surprisingly, applicant has discovered that LF can be stabilized and its antimicrobial activity increased, if the LF is immobilized by binding its N-terminus to a suitable naturally occurring substrate. (Application, page 7, lines 34- page 8, line 3.)

Claims 38 and 39 are directed to specific concentrations of immobilized lactoferrin on the surface of the composition subject to microbial contamination. Claims 149-151, 153, 164, 171-173, 175, 186, 193-195, and 197 are directed to a method for reducing the microbial contamination of a human or non-human vertebrate subject to microbial contamination using immobilized LF.

(6) Grounds of Rejection To Be Reviewed On Appeal

(i) Whether claims 102-104, 115-117, 119, 124, 127, 128, 137, 138, 142-148, 154, 157, 158, 171, 172, 176, 179, 180, 186, and 193-196 are unpatentable under 35 U.S.C. §102(e) as being anticipated by US 6,475,511 B2 ("Gohlke et al.").

(ii) Whether claims 1, 11, 19, 28, 31, 39, 101-102, 119-124, 126-129, 131, 132, 134, 142-148, 197, and 200 are unpatentable under 35 USC §102(b) as being anticipated by WO Patent Application 91/13982 ("WO Patent Application '982").

(iii) Whether claims 149-151, 153, 164, 171-173, 175, 186, and 193-195 are unpatentable under 35 USC §103(a) as being obvious in view of WO Patent Application 91/13982 ("WO Patent Application '982").

(iv) Whether claims 1, 2, 5, 18, 19, 22, 31, 101-103, 106, 115-117, 119-124, 126-129, 131-132, 134, 136, 142-151, 153, 164, 171-173, 175, 186, 193-197, and 200-202 are unpatentable under 35 USC § 102(b) as being anticipated by European Patent Application 753,309 ("European Patent Application '309").

(v) Whether claims 38 and 39 are unpatentable under 35 USC §103(a) as being obvious in view of European Patent Application 753,309 ("European Patent Application '309").

(vi) Whether claims 1, 2, 5, 18, 19, 22, 31, 32, 101-103, 106, 115, 119-124, 126-129, 131-136, 142-151, 153, 159, 162-165, 171-173, 175, 181, 184-187, 193-197, and 200-202 are unpatentable under 35 USC §102(b) as being anticipated by European Patent Application 753,308 ("European Patent Application '308").

(vii) Whether claims 38 and 39 are unpatentable under 35 USC §103(a) as being obvious in view of European Patent Application 753,308 ("European Patent Application '308").

(viii) Whether claims 1-3, 5, 18-20, 22, 31, 32, 102-104, 106, 115, 119, 124, 137, 138, 142-150, 154, 164, and 165 are unpatentable under 35 USC §102(e) as being anticipated by US Patent 6,066,469 by Kruzel et al. ("Kruzel et al.").

(7) Argument

I. Summary of Argument

The appealed claims all require, *inter alia*, the immobilization of lactoferrin on a naturally occurring substrate via the lactoferrin's N-terminus region. The examiner relies on a number of references which are alleged to inherently disclose such immobilized lactoferrin. However, the examiner provides no evidence that such immobilization occurs and, consequently, has failed to establish the requisite prima facie basis for the rejections.

In marked contrast, applicant has provided factual evidence, in the form of the declaration of Dr. Andrew Barron, establishing that none of the references relied on by the examiner inherently discloses the formation of lactoferrin immobilized on a naturally occurring substrate via the lactoferrin's N-terminus region. As explained by Dr. Barron, the reasons that immobilization cannot occur are because (1) the "substrates" are too small to immobilize lactoferrin, (2) the "substrates" do not possess the proper charge to bind lactoferrin's positively charged N-terminus, or (3) the proper conditions for immobilization are not described.

Because one or more of Applicant's arguments apply to each of the rejected claims, Applicant has grouped the claims. For the purpose of this appeal, the claims within each of the following groups will stand or fall together.

Group 1: 1, 2, 5, 18, 19, 22, 31, 38, 39, 104, 106, 120, 121, 122, 123, , 129, 131, 132, 134, 136, 149, 150, 151, 153, 162, 163, 164, 165, 173, 175, 186, 197, 200, 201, and 202.

Group 2: 102, 103, 115, 116, 117, 119, 127, 128, 142, 143, 144, 145, 146, 147, 148, 171, 172, 193, 194, 195, and 196.

Group 3: 104, 137, 138, 154, 157, 158, 176, 179, and 180.

Group 4: 133, 135, 159, 181, 184, 185, and 187.

Group 5: 11 and 28.

Group 6: 20.

Group 1 consists of the claims that are distinguished over the references cited against them by Applicant's argument numbered 2) above. Group 2 consists of the claims that are

distinguished over the references cited against them by Applicant's arguments numbered 1) and 3) above. Group 3 consists of the claims that are distinguished over the references cited against them by Applicant's argument numbered 3) above. Group 4 consists of the claims that are distinguished over the references cited against them by Applicant's arguments numbered 1) or 2) above. Group 5 consists of the claims that are distinguished over the references cited against them by Applicant's arguments numbered 1) or 2) or 3) above. Group 6 consists of the claims that are distinguished over the references cited against them by Applicant's arguments numbered 1) or 3) above.

II. Examiner's Burden

The examiner bears the burden of establishing a prima facie basis for each ground for rejection of the claims on appeal. In re Alton, 76 F.3d 1168, 1175 (Fed. Cir. 1996); In re Oetiker, 977 F.2d 1443, 1445 (Fed. Cir. 1992). With respect to the core factual findings in a determination of a lack of patentability, the examiner cannot simply reach conclusions based on his own understanding or experience or on his assessment of what would be basic knowledge of common sense. In re Zurko, 258 F.3d 1379, 1386 (Fed. Cir. 2001.) Instead, the examiner must point to some concrete evidence in the record to support the findings underlying the rejections. (Id.) As stated in Continental Can. Co. USA, Inc. v. Monsanto, 948 F.2d 1264, 1268 and 1269 (Fed. Cir. 1991):

> "To serve as anticipation when the reference is silent about the asserted inherent characteristic, such gap in the reference may be filled with recourse to extrinsic evidence. Such evidence must make clear that the missing descriptive matter is necessarily present in the thing described in the reference, and that it would be so recognized by persons of ordinary skill. In re Oelrich, 666 F.2d 578, 581, 212 U.S.P.Q. 323, 326 (C.C.P.A. 1981)

(Bold added.)

Continental Can was cited with approval in In re Robertson, 169 F.3d 743, 745 (Fed. Cir. 1999), where the court explained:

> "To establish inherency, the extrinsic evidence 'must make clear that the missing descriptive matter is necessarily present in the thing described in the reference, and that it would be so recognized by persons of ordinary skill.' Continental Can. Co. v. Monsanto Co., 948 F.2d 1264, 1268, 20 U.S.P.Q.2d 1746, 1749 (Fed. Cir. 1991). 'Inherency, however, may not be established by probabilities or possibilities. The mere fact that a certain thing may result from a given set of circumstances is not sufficient.' Id. At 1269, 948 F.2d 1264, 20 U.S.P.Q.2d at 1749 (quoting In re Oelrich, 666 F.2d 578, 581, 212 U.S.P.Q. 323, 326 (C.C.P.A. 1981))."

(Bold added.)

The examiner has failed to provide any such concrete evidence to support his assertions that the references inherently disclose lactoferrin immobilized on a naturally occurring substrate via the lactoferrin's N-terminus region and, therefore, the rejections should be withdrawn.

III. <u>Declaration of Dr. Andrew R. Barron</u>

Applicant submitted the Declaration of Dr. Andrew R. Barron, under 37 C.F.R. §1.132, on August 24, 2004. Dr. Barron received A.R.C.S. and B.Sc. (1st Class, Hones.) degrees, majoring in chemistry, at Imperial College of Science and Technology, University of London in 1986. (Barron Decl., ¶ 1.) He received a D.I.C. and Ph.D., at the same university in 1986. (Id.) He was a Post-Doctoral Research Associate, at the University of Texas, Austin in 1986-1987. (Id.)

Dr. Barron was an Assistant Professor and then an Associate Professor, at Harvard University from 1987 to 1995. (Barron Decl., ¶ 2.) In the fall of 1995, he went to Rice University, where he is currently the Charles W. Duncan, Jr. - Welch Chair of Chemistry and Professor of Materials Science in the Department of Chemistry and Department of Mechanical Engineering and Materials Science. (Barron Decl., ¶ 3.) He has authored over two hundred journal articles and has made a like number of presentations. (Barron Decl., ¶ 4.)

Lactoferrin (LF) is a protein (or peptide). (Specification, page 11, lines 13 and 14.) A full length LF peptide sequence has about 600 to about 800 continuous amino acids. (Barron Decl., ¶ 7.) Human LF, in particular, is about 703 amino acids long and has a molecular weight of about 83,000 daltons. (Id.) Furthermore, as explained by Dr. Barron, LF has a bilboate structure, with a positively charged N-terminus lobe and a negatively charged C-terminus lobe. (Id.)

LF is a known antimicrobial agent. (Specification, col. 8, lines 10 and 11.) Its activity is highly dependent on its three-dimensional or tertiary structure. (Specification, col. 8, lines 23 and 24.) If LF does not have the proper conformation, its activity is diminished or lost. Specification, col. 8, lines 24 and 25.)

Now it has been unexpectedly found that LF can be stabilized and its antimicrobial activity increased, if the LF is immobilized by binding its N-terminus to a suitable, naturally occurring substrate, i.e., if LF has its N-terminus region attached to a substrate leaving the C-terminus region free to interact with microbes. (Appl. page 7, line 34-page 8, line 3.) Consequently, among the requirements for LF to become immobilized on a substrate and, in particular on a naturally occurring substrate, the portion of the substrate which is to do the binding should carry the opposite charge, i.e., carry a negative charge. (Barron Decl., ¶ 9.)

The examiner discounts Dr. Barron's declaration. The examiner asserts that the declaration should be ignored, because Dr. Barron "performs no direct testing on the prior art compositions in order to determine whether or not they comprise LF immobilized on a naturally occurring substrate via the N-terminus region of the LF," but "attempt[s] to show by scientific reasoning and/or argument that the prior art compositions [sic] do not teach this feature."

Applicant disagrees with the examiner's refusal to consider Dr. Barron's declaration. Dr. Barron's declaration must be considered and it is persuasive on the issue of whether any of the references relied upon by the examiner inherently disclose LF immobilized on a naturally occurring substrate via the N-terminus region of the LF. Dr. Barron's declaration has a well founded factual basis that is entirely consistent with the disclosure set forth in applicant's

specification. There is no requirement that a declaration submitted under 37 C.F.R. 1.132 must be based on direct testing. For example, in In re Alton, 76 F.3d at 1175, the Federal Circuit held that the examiner had erred in dismissing a declaration based on "statements of fact."

The examiner further asserts that:

> "One major argument made by Declarant and in the Remarks is that '[f]or the N-terminus region to become immobilized on a naturally occurring substrate, the region of the substrate to which the N-terminus region is to become attached should carry the opposite charge, i.e., carry a negative charge. . .' However, Declarant does not provide any citation to the specification which would support this contention, and the examiner can find no support in the original disclosure of the invention for this contention."

Support for the contention is provided by applicant's frequent and unequivocal teaching that LF must be immobilized on the substrate via the N-terminus region of the LF. Applicant was not required to provide an explanation in the specification concerning how his invention worked, i.e., how the N-terminus region is immobilized on the substrate. Newman v. Quigg, 877 F.2d 1575, 1581 (Fed. Cir. 1989). Dr. Barron's explanation – that to be immobilized, the positive N-terminus region must bind with a negatively charged substrate – is of importance only in the context of subsequently trying to help the examiner understand how the invention is distinguishable over the prior art.

The examiner additionally argues that:

> "Further, this argument is inconsistent with the disclosure in the specification of useful substrates which do not have a positive charge. For example, the original specification at page 10, line 22, and originally-filed claim 3 disclose triglycerides to be useful substrates for immobilizing lactoferrin by its N-terminus region. Triglycerides are uncharged. The original specification at page 10, lines 19-22, and originally-filed claim 3 disclose proteins, polysaccharides, and lipids to be useful substrates for immobilizing

lactoferrin by its N-terminus. These classes of compounds embrace positively charged, negatively charged, and uncharged compounds."

However, what is clear when the application is considered in its entirety is that a suitable substrate must be one on which the LF becomes immobilized via its N-terminus. For example, originally filed dependent claim 3 did not simply, cover specific substrates. Instead, the substrates were subject to the limitation that the LF must be immobilized via its N-terminal region. For the reasons given by Dr. Barron, LF cannot bind to triglycerides and other lipids via its N-terminal region. (It is important to note that there is nothing in the record that contradicts Dr. Barron's declaration. None of the references relied upon by the examiner suggest that LF can become immobilized on lipids, such as triglycerides.) Consequently, what is inconsistent with applicant's overall teachings is applicant's initial recitation of triglycerides and other lipids – not Dr. Barron's declaration.

Similarly, applicant has never stated that all proteins and polysaccharides are useful. They too are subject to the limitation that the LF must be immobilized on the substrate via its N-terminal region. There are numerous examples of suitable proteins and polysaccharides. Useful proteins include fibronectin, casein and mucin, while suitable polysaccharides include galactose-rich polysaccharides, collagen, heparin-sulfate, and carrageenan. (Page 10, lines 19-23.) These all contain negatively charged regions and, accordingly, are all consistent with Dr. Barron's explanation of what it takes to immobilize LF by its N-terminus region. Therefore, Dr. Barron's declaration is not inconsistent with the claimed invention.

Still further, the examiner asserts:

> "Finally, this argument by Declarant uses a significant qualifier "should." Because of this use of this word, Declarant in effect admits that a substrate does not have to have a negative charge in order to be useful in immobilizing lactoferrin by its N-terminus."

Applicant respectfully disagrees. The primary definition of "should" as found in Merriam-Webster's Collegiate Dictionary, Eleventh Addition, p. 1153 is "1 -- used in auxiliary function to express condition <if he ~ leave his father, his father would die – Gen 44:22<RSV>" Dr. Barron uses "should" in this same conditional sense – if LF is to be bound to the substrate via its N-terminus, the substrate ~ have a region carrying a negative charge. Therefore, Dr. Barron's use of the word "should," supports the argument that LF must be immobilized on a substrate having a negative region.

IV. Gohlke et al. does not Anticipate the Claimed Invention

US 6,475,511 B2 ("Gohlke et al.") do not anticipate any of claims 102-104, 115-117, 119, 124, 127, 128, 137, 138, 142-148, 154, 157, 158, 171, 172, 176, 179, 180, 186, and 193-196 under 35 U.S.C. §102(e). Gohlke et al. describe compositions containing a combination of colostrum and LF in a "mucosal delivery format" ("MDF"). (Col. 6, lines 13-28.) The composition can also contain modified pectin. (Col. 6, lines 49-52.) By MDF is meant a composition, such as a lozenge, formed of solid components. (Barron Decl., ¶ 11.) For example, Gohlke et al. teach, "The individual components of the composition may be obtained from commercial sources: colostrum (which is dehydrated by standard spray-drying procedures known in the art)" (col. 9, lines 41-44). Furthermore, examples 1 - 3 describe a process for preparing the compositions where, "[E]ach of the following ingredients is placed, in powdered form, into a commercial mixer." (Emphasis added.) The ingredients are then mixed and cold pressed.

A. Gohlke et al. does not Teach the Formation of Immobilized LF

The examiner argues that:

"With respect to Gohlke et al (U.S. Patent No. 6,475,511), Declarant argues that cold pressing as occurs in Gohlke et al will not provide an environment suitable to cause lactoferrin to become attached to the colostrum or the modified pectin via the lactoferrin's N-terminus region. However, Declarant does not provide any reasoning or

evidence as to why these processing steps of Gohlke et al are insufficient to result in immobilization via the N-terminus of lactoferrin.

> "Further, there is no disclosure anywhere in the specification that special procedures or conditions are necessary in order to achieve the desired immobilization. See, e.g., page 11, lines 3-11, of the specification. In the absence of a disclosed need for special conditions, Gohlke et al's disclosed thorough mixing and cold pressing of the ingredient in powder form is deemed to be sufficient to result in the claimed immobilization."

(Emphasis added.)

However, Dr. Barron makes plain that immobilized LF will not be formed, if LF is simply admixed with another solid. He explains, "The mere presence of LF in a cold-pressed mixture with other solids, such as colostrum and modified pectin in an MDF format, would not inherently result in the LF becoming attached via its N-terminus." (Barron Decl., ¶ 13, emphasis added.)

Furthermore, it is not clear what the examiner means by "special conditions." Applicant teaches that LF is immobilized on the substrate using a suitable technique and gives as an example "mixing the LF with the biologically active substrate in a suitable medium, such as deionized water." (Specification, page 11, lines 3-5.) Accordingly, nothing in the specification, in Gohlke et al. or in any of the other references contradicts Dr. Barron's declaration and suggests that LF can become immobilized simply by admixing with another solid. Therefore, Gohlke et al. does not anticipate any of claims 102-104, 115-117, 119, 124, 127, 128, 137, 138, 142-148, 154, 157, 158, 171, 172, 176, 179, 180, 186, and 193-196, so that this ground for rejection should be withdrawn.

V. <u>WO Patent Application '982 does not Anticipate the Claimed Invention</u>

WO Patent Application 91/13982 ("WO Patent Application '982") does not anticipate any of claims 1, 2, 11, 18, 19, 28, 31, 39, 101-103, 119-124, 126-129, 131, 132, 134, 142-148, 197,

and 200 under 35 USC §102(b). WO Patent Application '982 generally relates to human LF expressed using recombinant DNA. It discloses the use of this LF as a nutritional supplement, an antiseptic, and as a food-spoilage retardant. The LF can be compounded with certain carriers or diluents.

WO Patent Application '982 neither broadly teaches LF immobilized on a naturally occurring substrate via the N-terminus region of the LF, nor does it provide a specific example of such an immobilized LF. (Barron Decl., ¶ 15.) The examiner suggests that:

"The WO Patent Application '982 teaches LF in combination with stearic acid (which is a lipid and also corresponds to Applicant's pharmaceutically acceptable carrier of claim 102) or its salts . . . Because the same components are present in the same defined dispersion, inherently the LF in the composition of the WO Patent Application '982 will be immobilized via its N-terminus. . ."

A. <u>WO Patent Application '982 does not Teach the Formation of Immobilized LF</u>

The mere presence in a mixture of LF and stearic acid would not form LF immobilized on LF via its N-terminus. WO Patent Application '982 does not disclose or suggest any conditions under which the compounds could be mixed to achieve such immobilization. Merely compounding solid LF with solid stearic acid, such as by cold-pressing the solid ingredients, will not provide an environment suitable to cause the LF to become immobilized via its N-terminus region. Instead, appropriate conditions must be chosen before immobilization can occur. As described in the instant application, LF is immobilized by first mixing the LF with the naturally occurring substrate in a suitable medium, such as deionized water.

The examiner asserts that:

"Declarant also argues that mere compounding will not result in lactoferrin's attachment to the stearic acid through the N-terminus of the lactoferrin. However, Declarant does not provide any reasoning or evidence to support this argument, and there

> is not disclosure any where (sic) in the specification that special procedures or conditions are necessary in order to achieve the desired immobilization. Further, because stearic acid has a negatively charged carboxyl group, all that it would take for the positively charged N-terminus of lactoferrin to become immobilized on the negatively charged carboxyl group would be to bring the two opposite charges into close physical proximity – charge attraction will do the remainder of the work. Any pharmaceutical compounding step will provide the necessary physical proximity so that at least some of the lactoferrin is immobilized by its N-terminus to a (sic) least some of the stearic acid.

(Emphasis added.)

On the contrary, Dr. Barron makes plain that simply compounding LF with stearic acid will not result in LF's attachment. As Dr. Barron explains, "Merely compounding solid LF with other solids, such as stearic acid, will not provide an environment suitable to cause the LF to become attached to the other solid via LF's N-terminus region." (Barron Decl., ¶ 11.) The basis for his reasoning is readily apparent, merely admixing two solids, even if they have regions of opposing charges, does not provide the conditions necessary for immobilization to occur.

Furthermore, it is not clear what is meant by "special procedures or conditions". Applicant teaches that LF is immobilized on the substrate using a suitable technique and gives as an example "mixing the LF with the biologically active substrate in a suitable medium, such as deionized water." (Specification, page 11, lines 3-5.) Accordingly, nothing in the specification, in WO Patent Application '982 or any of the other references relied upon by the examiner contradicts Dr. Barron's declaration and suggests that LF can become immobilized by using "any pharmaceutical compounding step."

B. WO Patent Application '982 does not Disclose any Compounds having the Size Required of a Substrate in order to Immobilize LF

Moreover, stearic acid, with a molecular weight of only 284.47, is too small to be used as a naturally occurring substrate as the term is to be understood in the context of the instant specification and claims. That would be akin to saying that a dog was immobilized on a flea, if a flea attached itself to a dog.

The examiner asserts that:

> "This argument cannot be accepted because it contradicts the original disclosure of substrates with molecular weights significantly less than that of lactoferrin. For example, page 10, lines 19-22, of the specification and originally-filed claim 3 recite that nucleic acids, nucleotides, lipids, adenosine triphosphate, and triglycerides are useful and acceptable substrates. These exemplified substrates have molecular weights which are significantly less than that of lactoferrin. Accordingly, stearic acid can not be disqualified as a substrate merely because of its molecular weight."

What is wrong, however, is not applicant's argument, as supported by Dr. Barron's declaration. Instead, what was wrong was the original inclusion of lipids, such as stearic acid, among the examples of suitable substrates. The reality is that LF cannot be immobilized by a lipid. As explained by Dr. Barron, "Stearic acid with a molecular weight of only 284.47 is not a substrate. LF could not become immobilized on such a small molecule." (Barron Decl., ¶ 17.) Therefore, the rejection of claims 1, 2, 11, 18, 19, 28, 31, 39, 101-103, 119-124, 126-129, 131, 132, 134, 142-148, 197, and 200 as obvious in view of WO Patent Application '982 should be withdrawn.

VI. WO Patent Application '982 Would not have Made Obvious the Claimed Invention

WO Patent Application 91/13982 ("WO Patent Application '982") would not have made obvious claims 149-151, 153, 164, 171-173, 175, 186, 193-195, and 197, the claims covering a

method for reducing the microbial contamination of a human or non-human vertebrate subject to microbial contamination. The examiner argues that:

> "The WO Patent Application '982 teaches administering its antiseptics to mammals, but does not particularly teach treating humans or non-human vertebrates. It would have been obvious to one of ordinary skill in the art at the time of Applicant's invention was made to use the antiseptic compositions of the WO Patent Application '982 to treat both human and non-human mammals . . ."

However, for the reasons discussed immediately above, WO Patent Application '982 would not have suggested immobilizing LF on a naturally occurring substrate via its N-terminus region. Therefore, the rejection of claims 149-151, 153, 164, 171-173, 175, 186, 193-195, and 197 as being obvious in view of WO Patent Application '982 should be withdrawn.

VII. <u>European Patent Application '309 does not Anticipate the Claimed Invention</u>

European Patent Application 753,309 ("European Patent Application '309") does not anticipate any of claims 1, 2, 5, 18, 19, 22, 31, 101-103, 106, 115-117, 119-124, 126-129, 131-132, 134, 136, 142-151, 153, 164, 171-173, 175, 186, 193-197, and 200-202 under 35 USC § 102(b). European Patent Application '309 generally relates to the preparation of mixtures of LF and desferrioxamine methanesulphonate useful for the therapy of viral infectious diseases.

European Patent Application '309 neither broadly teaches LF immobilized on a naturally occurring substrate via the N-terminus region of the LF, nor does it provides a specific example of such an immobilized LF. (Barron Decl., ¶ 21.) The examiner asserts that:

> "The European Patent Application '309 teaches compositions comprising LF and carriers such as paraffin oil and Vaseline (which are lipids), xantan gum and corn starch (which are polysaccharides), and lecithin (which is an emulsifier) .... Because the same components are present in the same defined

dispersion, inherently the LF in the composition of the European Patent Application '309 will be immobilized by its N-terminus . . ."

A. European Patent Application '309 does not Disclose any Compounds having the Size Required to Immobilize LF

Paraffin oil or, as it is alternatively called mineral oil, is a mixture of liquid hydrocarbons. (Concise Chemical and Technical Dictionary, H. Bennett, Ed., Chemical Publishing Co., Inc. (1974) (pp. 702 and 777)) Vaseline is a petroleum jelly, i.e., a purified mixture of semi-solid hydrocarbons. (Id., pp. 798 and 1100.) They are low molecular weight compounds, not substrates, and LF could not become immobilized on such small molecules. (Barron Decl., ¶ 23.)

Similarly, lecithin is a low molecular weight compound. (Barron Decl., ¶ 27.) LF could not become immobilized on such a small molecule. (Id.)

B. European Patent Application '309 does not Disclose any Compounds having the Charge Required to Immobilize LF

Additionally, paraffin oil and Vaseline are both hydrocarbons, so that they do not carry any charges. (Barron Decl., ¶ 25.) As a result, neither paraffin oil nor Vaseline contains a region which will attach LF's positively charged N-terminus region. (Id.)

Similarly, xanthan gum and corn starch do not carry any charges. (Barron Decl., ¶ 26.) As a result, neither xanthan gum nor corn starch contains a region which will attach LF's positively charged N-terminus region. (Id.)

Applicant's arguments are fully supported by Dr. Barron's declaration. Applicant has never asserted that all polysaccharides are useful. On the contrary, the polysaccharides are subject to the limitation that the LF must be immobilized to the substrate via its N-terminal region. Therefore, the claims do not read on low molecular weight compounds, such as paraffin

oil, Vaseline, and lecithin or on compounds lacking the negatively charged region needed to immobilize the N-terminus region of LF, such as paraffin oil, Vaseline, xanthan gum or corn starch. Therefore, the rejection of claims 1, 2, 5, 18, 19, 22, 31, 101-103, 106, 115-117, 119-124, 126-129, 131-132, 134, 136, 142-151, 153, 164, 171-173, 175, 186, 193-197, and 200-202 as being anticipated by European Patent Application '309 should be withdrawn.

VIII. <u>European Patent Application '309 Would not have Made Obvious the Claimed Invention</u>

European Patent Application '309 would not have made obvious claims 38 or 39, the claims additionally specifying the concentration of lactoferrin on the surface of the composition subject to microbial contamination, under 35 USC § 103(a).

The examiner argues that:

> "The European Patent Application '309 does not teach a lactoferrin/surface area ratio for surfaces to be treated. It would have been obvious to one of ordinary skill in the art at the time Applicant's invention was made to determine all operable and optimal does for the lactoferrin-containing compositions . . ."

However, for the reasons discussed immediately above, European Patent Application '309 would not have suggested immobilizing LF on a naturally occurring substrate via its N-terminus region. Therefore, European Patent Application '309 would not have made obvious either claim 38 or 39, so that this ground for rejection should be withdrawn.

IX. <u>European Patent Application '308 does not Anticipate the Claimed Invention</u>

European Patent Application 753,308 ("European Patent Application '308") does not anticipate any of claims 1, 2, 5, 18, 19, 22, 31, 32, 101-103, 106, 115, 119-124, 126-129, 131-136, 142-151, 153, 159, 162-165, 171-173, 175, 181, 184-187, 193-197, and 200-202 under 35 USC §102(b). European Patent Application '308 generally relates to the use of LF for therapy of diseases caused by Gram positive pathogen microorganisms.

European Patent Application `308 neither broadly teaches LF immobilized on a naturally occurring substrate via the N-terminus region of the LF, nor does it provide a specific example of such an immobilized LF. (Barron Decl., ¶ 26.) The examiner asserts that:

> "The European Patent Application '308 teaches compositions comprising LF and peppermint oil, gum base and corn starch (which are polysaccharides) . . . Because the same components are present in the same defined dispersion, inherently the LF in the composition of the European Patent Application '308 will be immobilized via its N-terminus . . ."

A. <u>European Patent Application '308 does not Disclose any Compounds having the Size Required to Immobilize LF</u>

Peppermint oil is a low molecular weight compound. LF could not become immobilized on such a small molecule. (Barron Decl., ¶ 28.)

B. <u>European Patent Application '308 does not Disclose any Compounds having the Charge Required to Immobilize LF</u>

Furthermore, peppermint oil, gum base and corn starch do not carry any charges. As a result, neither peppermint oil, gum base nor corn starch contain a region which will attach LF's positively charged N-terminus region.

Applicant's arguments are fully supported by Dr. Barron's declaration. Applicant has never asserted that all polysaccharides are useful. On the contrary, the polysaccharides are subject to the limitation that the LF must be immobilized to the substrate via its N-terminal region. Therefore, the claims do not read on low molecular weight compounds, such as peppermint oil or on compounds lacking the negatively charged region needed to immobilize the N-terminus region of LF, such as gum base or corn starch. Therefore, the rejection of claims 1, 2, 5, 18, 19, 22, 31, 32, 101-103, 106, 115, 119-124, 126-129, 131-136, 142-151, 153, 159,

162-165, 171-173, 175, 181, 184-187, 193-197, and 200-202 as being anticipated by European Patent Application '308 should be withdrawn.

X. European Patent Application '308 Would not have Made Obvious the Claimed Invention

European Patent Application '308 would not have made obvious claims 38 or 39, the claims specifying the concentration of lactoferrin on the surface of the composition subject to microbial contamination, under 35 USC § 103(a).

The examiner argues that:

> "The European Patent Application '308 does not teach a lactoferrin/surface area ratio for surfaces to be treated. It would have been obvious to one of ordinary skill in the art at the time Applicant's invention was made to determine all operable and optimal does for the lactoferrin-containing compositions . . ."

However, for the reasons discussed immediately above, European Patent Application '308 would not have suggested immobilizing LF on a naturally occurring substrate via its N-terminus region. Therefore, European Patent Application '308 would not have made obvious either claim 38 or 39, so that this ground for rejection should be withdrawn.

XI. Kruzel et al. Do not Anticipate the Claimed Invention

US Patent 6,066,469 by Kruzel et al. ("Kruzel et al.") does not anticipate any of claims 1-3, 5, 18-20, 22, 31, 32, 102-104, 106, 115, 119, 124, 137, 138, 142-150, 154, 164, and 165 under 35 USC § 102(e). Kruzel et al. disclose the use of LF as a nutritional supplement, as an antiseptic to treat and prevent opportunistic bacterial, viral and fungal infections, and as a food-spoilage retardant. Kruzel et al neither broadly teach LF immobilized on a naturally occurring substrate via the N-terminus region of the LF, nor provide a specific example of such an immobilized LF. (Barron Decl., ¶ 30.) The examiner asserts that:

"Kruzel et al. teach nutritional supplements comprising LF in combination with adjuvants or diluents such as cellulose, starch, tragacanth, and sodium carboxymethlycellulose . . . . Because the same components are present in the same defined dispersion, inherently the LF in the nutritional supplements of Kruzel et al will be immobilized via its N-terminus . . ."

A. <u>Kruzel et al. does not Teach the Formation of Immobilized LF</u>

Dr. Barron makes plain that Kruzel et al. does not disclose nor suggest any conditions under which the compounds could be mixed to result in the LF becoming attached via its N-terminus. (Barron Decl., ¶ 35.) He explains, "The mere presence in a mixture of LF and an adjuvant or a diluent, such as the solids cellulose, starch, tragacanth, and sodium carboxymethlycellulose would not inherently result in the LF becoming attached via its N-terminus." (Barron Decl., ¶ 33.)

B. <u>Kruzel et al does not Disclose any Compounds having the Charge Required to Immobilize LF</u>

Cellulose and starch do not carry any charges. (Barron Decl., ¶ 34.) As a result, neither cellulose nor starch contains a region which will immobilize LF's positively charged N-terminus region. (Id.)

Applicant's argument is fully supported by Dr. Barron's declaration. Applicant has never asserted that all naturally occurring "substrates" are useful. On the contrary, the substrates are subject to the limitation that the LF must be immobilized via its N-terminal region. Therefore, the claims do not read on compounds lacking the negatively charged region needed to immobilize the N-terminus region of LF, such as cellulose or starch. Therefore, the rejection of claims 1-3, 5, 18-20, 22, 31, 32, 102-104, 106, 115, 119, 124, 137, 138, 142-150, 154, 164, and 165 as being anticipated by Kruzel et al. should be withdrawn.

XII. Conclusion

For the foregoing reasons, Claims 1, 2, 5, 11, 18-20, 22, 28, 31, 32, 38, 39, 86, 101-104, 106, 115-117, 119-124, 126-129, 131-138, 142-151, 153, 154, 157-159, 162-165, 171-173, 175, 176, 179-181, 184-187, 193-197, and 200-202, all of the claims on appeal, should be found allowable.

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Respectfully submitted,

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(8) Claims Appendix (From Applicant's Reply to January 7, 2005 Office Action)

1. A composition of matter comprising a dispersion of isolated lactoferrin immobilized on a naturally occurring substrate not including gelatin via the N-terminus region of the lactoferrin.

2. The composition in accordance with claim 1, wherein the naturally occurring substrate not including gelatin is a protein, a polysaccharide, a nucleic acid, or a nucleotide.

3. The composition in accordance with claim 1, wherein the naturally occurring substrate not including gelatin is collagen, fibronectin, casein, mucin, heparan-sulfate, carrageenan, deoxyribonucleic acid, or adenosine triphosphate.

4. The composition in accordance with claim 1, wherein the naturally occurring substrate not including gelatin is a galactose-rich polysaccharide comprising mainly galactose residues and derivatized galactose residues.

5. The composition of claim 1, wherein the dispersion is an aqueous solution, an aqueous emulsion, a colloid, a suspension, a powder, or a granular solid.

6. A composition of matter comprising a dispersion of isolated lactoferrin immobilized on a naturally occurring substrate via the N-terminus region of the lactoferrin, and native lactoferrin.

7. The composition in accordance with claim 6, wherein the concentration of immobilized lactoferrin and native lactoferrin in the dispersion is from about 0.05% wt/vol to about 2.5 % wt/vol.

8. The composition in accordance with claim 6, wherein the molar ratio of immobilized lactoferrin to native lactoferrin is a ratio of from about 1:1 to about 1:10.

9. The composition in accordance with claim 6, wherein the molar ratio of immobilized lactoferrin to native lactoferrin is a ratio of from about 1:1 to about 1:5.

10. The composition in accordance with claim 6, wherein the composition comprises about 1 % wt/vol immobilized lactoferrin and about 1 % wt/vol native lactoferrin.

11. The composition in accordance with claim 1, wherein the composition further comprises a buffer system.

12. The composition in accordance with claim 11, wherein the buffer system contains a physiologically acceptable acid, a physiologically acceptable base, and a physiologically acceptable salt.

13. The composition in accordance with claim 12, wherein the physiologically acceptable acid is oxalic acid, ethylenediamine tetraacetic acid, carbonic acid, or citric acid; the physiologically acceptable base is sodium bicarbonate, potassium bicarbonate, sodium carbonate, or potassium carbonate; and the physiologically acceptable salt is calcium chloride, potassium chloride or sodium chloride.

14. A composition of matter comprising an aqueous buffer solution containing a physiologically acceptable acid selected from the group consisting of oxalic acid, ethylenediamine tetraacetic acid, carbonic acid, and citric acid; a physiologically acceptable base; and a physiologically acceptable salt selected from the group consisting of calcium chloride, potassium chloride, and sodium chloride, wherein the ratio of acid to base to salt is 0.1 to 0.0001 M (acid) : 1 to 0.001 M (base) : 10 to 0.01M (salt) and containing a mixture of native lactoferrin and isolated lactoferrin immobilized on a galactose-rich polysaccharide comprising mainly galactose residues and derivatized galactose residues, collagen, fibronectin, casein, mucin, heparan-sulfate, carrageenan, deoxyribonucleic acid, or adenosine triphosphate via the N-terminus region of the lactoferrin, in a native lactoferrin to isolated immobilized lactoferrin molar ratio of from about 1:1 to about 1:5 and in a concentration of from about 0.001 to about 2.5 % wt/vol.

15. The composition in accordance with claim 14, wherein the lactoferrin is immobilized on a galactose-rich polysaccharide comprising mainly galactose residues and derivatized galactose residues.

16. The composition in accordance with claim 14, wherein the mixture comprises about 1 % wt/vol immobilized lactoferrin and about 1% wt/vol native lactoferrin.

17. The composition in accordance with claim 14, wherein the physiologically acceptable acid is citric acid, the physiologically acceptable base is sodium bicarbonate and the physiologically acceptable salt is sodium chloride.

18. A method for reducing the microbial contamination of a composition subject to microbial contamination by a microbe, comprising: treating the composition with a sufficient amount of isolated lactoferrin immobilized on a naturally occurring substrate not including gelatin via the N-terminus region of the lactoferrin to reduce microbial contamination.

19. The method in accordance with claim 18, wherein the naturally occurring substrate, not including gelatin, is a protein, a polysaccharide, a nucleic acid, or a nucleotide.

20. The method in accordance with claim 18, wherein the naturally occurring substrate not including, gelatin is collagen, fibronectin, casein, mucin, heparan-sulfate, carrageenan, deoxyribonucleic acid, or adenosine triphosphate.

21. The method in accordance with claim 18, wherein the naturally occurring substrate, not including gelatin, is a galactose-rich polysaccharide comprising mainly galactose residues and derivatized galactose residues.

22. The method of claim 18, wherein the composition is an aqueous solution, an aqueous emulsion, a colloid, a suspension, a powder, or a granular solid.

23. The method in accordance with claim 18, further comprising applying a composition containing a mixture of immobilized lactoferrin and native lactoferrin.

24. The method in accordance with claim 23, wherein the concentration of the mixture in the composition is from about 0.001 to about 2.5% wt/vol.

25. The method in accordance with claim 23, wherein the molar ratio of immobilized lactoferrin to native lactoferrin in the mixture is in a ratio of from about 1:1 to about 1:10.

26. The method in accordance with claim 23, wherein the molar ratio of immobilized lactoferrin to native lactoferrin in the mixture is in a ratio of from about 1:1 to about 1:5.

27. The method in accordance with claim 23, wherein the mixture comprises about 1 % wt/vol immobilized lactoferrin and about 1 % wt/vol native lactoferrin.

28. The method in accordance with claim 22, wherein the aqueous solution further comprises a buffer system.

29. The method in accordance with claim 28, wherein the buffer system contains a physiologically acceptable acid, a physiologically acceptable base, and a physiologically acceptable salt.

30. The method in accordance with claim 29, wherein the physiologically acceptable acid is oxalic acid, ethylenediamine tetraacetic acid, carbonic acid, or citric acid; the physiologically acceptable base is sodium bicarbonate, potassium bicarbonate, sodium carbonate, or potassium carbonate; and the physiologically acceptable salt is calcium chloride, potassium chloride or sodium chloride.

31. The method of claim 18, wherein the microbe is a bacterium, a fungus, a protozoan, or a virus.

32. The method in accordance with claim 18, wherein the microbe is enterotoxigenic Escherichia coli, enteropathogenic Escherichia coli, Shigella dysenteriae, Shigella flexneri,

Salmonella typhimurium, Salmonella typhi, Salmonella abony, Salmonella dublin, Salmonella enteritidis, Salmonella hartford, Salmonella kentucky, Salmonella panama, Salmonella pullorum, Salmonella rostock, Salmonella thompson, Salmonella virschow, Enterobacter aerogenes, Vibrio cholerae, Yersinia enterocolitica, Campylobacter jejuni, Aeromonas hydrophila, Staphylococcus aureus, Staphylococcus hyicus, Staphylococcus epidermidis, Staphylococcus hominis, Staphylococcus warneri, Staphylococcus xylosus, Staphylococcus chromogenes, Streptococcus pyogenes, Streptococcus pneumoniae, Streptococcus mutans, Streptococcus sanguis, Pediococcus acne, Bacillus cereus, Bacillus anthracis, Bacillus subtilis, a Brucella species, Listeria monocytogenes, Bordetella pertussis, Pseudomonas aeruginosa, Legionella pneumophila, Francisella tularensis, Candida albicans, Brochothrix thermospacta, Bacillus pumilus, Enterococcus faecium, Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Deinococcus radiopugnans, Deinococcus radiodurans, Deinobacter grandis, Acinetobacter radioresistens, or Methylobacterium radiotolerans.

33. The method in accordance with claim 18, wherein the microbe is a verotoxic Escherichia coli.

34. The method in accordance with claim 33, wherein the verotoxic Escherichia coli is the serotype 0157:H7.

35. The method of Claim 18, wherein the microbe is a Clostridium species.

36. The method of Claim 35, wherein the species is Clostridium perfringens, Clostridiumdifficile, Clostridiumbotulinum, or Clostridium tetani.

37. The method of Claim 18, wherein the microbe is a protozoan selected from the group consisting of Entamoeba histolytica, Naegleria flowleri, Giardia lamblia, Leishmania spp., Trichomonas vaginalis, Trypanosoma spp., Plasmodium spp., and Taxoplasma spp.

38. The method in accordance with claim 18, wherein the concentration of lactoferrin on the surface of the composition subject to microbial contamination is from about 0.0001 to about 10 mg /sq.inch.

39. The method in accordance with claim 38, wherein the concentration of lactoferrin on the surface of the composition subject to microbial contamination is from about 0.01 to about 1 mg/sq. inch.

40. A method for inhibiting the microbial contamination of a composition subject to microbial contamination comprising treating the composition with an aqueous buffer solution containing a physiologically acceptable acid selected from the group consisting of oxalic acid, ethylenediamine tetraacetic acid, carbonic acid, and citric acid; a physiologically acceptable base; and a physiologically acceptable salt selected from the group consisting of calcium chloride, potassium chloride, and sodium chloride, wherein the ratio of acid to base to salt is 0.1 to 0.0001M (acid): 1 to 0.001 M (base): 10 to 0.01 M (salt) and containing a mixture of native

lactoferrin and isolated lactoferrin immobilized on a galactose-rich polysaccharide comprising mainly galactose residues and derivatized galactose residues, collagen, gelatin, fibronectin, casein, mucin, heparan-sulfate, carrageenan, deoxyribonucleic acid, or adenosine triphosphate via the N-terminus region of the lactoferrin, in a native lactoferrin to isolated immobilized lactoferrin molar ratio of from about 1:1 to about 1:5 and in a concentration of from about 0.001 to about 2.5 % wt/vol.

41. The method in accordance with claim 40, wherein the lactoferrin is immobilized on galactose-rich polysaccharide comprising mainly galactose residues and derivatized galactose residues.

42. The method in accordance with claim 40, wherein the mixture comprises about 1 % wt/vol immobilized lactoferrin and about 1 % wt/vol native lactoferrin.

43. The method in accordance with claim 40, wherein the physiologically acceptable acid is citric acid, the physiologically acceptable base is sodium bicarbonate and the physiologically acceptable salt is sodium chloride.

44. The method of claim 40, wherein the microbe is bacterium, a fungus, a protozoan, or a virus.

45. The method in accordance with claim 40, wherein the microbe is enterotoxigenic Escherichia coli, enteropathogenic Escherichia coli, Shigella dysenteriae, Shigella flexneri, Salmonella typhimurium, Salmonella typhi, Salmonella abony, Salmonella dublin, Salmonella enteritidis, Salmonella hartford, Salmonella kentucky, Salmonella panama, Salmonella pullorum, Salmonella rostock, Salmonella thompson, Salmonella virschow, Enterobacter aerogenes, Vibrio cholerae, Yersinia enterocolitica, Campylobacter jejuni, Aeromonas hydrophila, Staphylococcus aureus, Staphylococcus hyicus, Staphylococcus epidermidis, Staphylococcus hominis, Staphylococcus warneri, Staphylococcus xylosus, Staphylococcus chromogenes, Streptococcus pyogenes, Streptococcus pneumoniae, Streptococcus mutans, Streptococcccus sanguis, Pediococcus acne, Bacillus cereus, Bacillus anthracis, Bacillus subtilis, a Brucella species, Listeria monocytogenes, Legionella pneumophila, Bordetella pertussis, Pseudomonas aeruginosa, Francisella tularensis, Candida albicans, Brochothrix thermospacta, Bacillus pumilus, Enterococcus faecium, Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Deinococcus radiopugnans, Deinococcus radiodurans, Deinobacter grandis, Acinetobacter radioresistens, or Methylobacterium radiotolerans.

46. The method in accordance with claim 40, wherein the microbe is a verotoxic Escherichia coli.

47. The method in accordance with claim 46, wherein the verotoxic Escherichia coli is the serotype 0157:H7.

48. The method of Claim 40, wherein the microbe is a Clostridium sp.

49. The method of Claim 48, wherein the species is Clostridium perfringens, Clostridium difficile, Clostridium botulinum, or Clostridium tetani.

50. CANCELLED

51. The method in accordance with claim 40, wherein the ratio of acid to base to salt is 0.01 to 0.001 M (acid) : 0.1 to 0.01 M (base) : 1 to 0.1 M(salt).

52. CANCELLED

53. CANCELLED

54. CANCELLED

55. CANCELLED

56. The method in accordance with claim 18, wherein the composition subject to microbial contamination is a foodstuff.

57. The method in accordance with claim 56, wherein the foodstuff is a meat product.

58. The method of claim 57, wherein the meat product is a beef product, a pork product, or a poultry product.

59. The method in accordance with claim 40, wherein the composition subject to microbial contamination is a foodstuff.

60. The method in accordance with claim 59, wherein the composition is a meat product.

61. The method of Claim 60, wherein the meat product is a beef product, a pork product, or a poultry product.

62. The method of claim 57, wherein the meat product is veal, lamb, sheep, goat, elk, deer, antelope, horse, or dog.

63. The method of claim 60, wherein the meat product is veal, lamb, sheep, goat, elk, deer, antelope, horse, or dog.

64. The method of claim 56, wherein the foodstuff comprises a surface and/or flesh of a marine or freshwater aquatic organism.

65. The method of claim 64, wherein the aquatic organism is a fish, mollusk, or crustacean.

66. The method of claim 59, wherein the foodstuff comprises a surface and/or flesh of a marine or freshwater aquatic organism.

67. The method of claim 66, wherein the aquatic organism is a fish, mollusk, or crustacean.

68. The method of claim 56, wherein the foodstuff comprises a vegetable foodstuff.

69. The method of claim 59, wherein the composition comprises a vegetable foodstuff.

70. A method for reducing the microbial contamination of a meat product subject to microbial contamination by a microbe, comprising: applying to the meat product a composition containing a physiologically acceptable acid selected from the group consisting of oxalic acid, ethylenediamine tetraacetic acid, carbonic acid, and citric acid; a physiologically acceptable base; and a physiologically acceptable salt selected from the group consisting of calcium chloride, potassium chloride, and sodium chloride, wherein the molar ratio of acid to base to salt is 0.1 to 0.0001 (acid) : 1 to 0.001 (base) : 10 to 0.01 (salt) and containing a mixture of native lactoferrin and isolated lactoferrin immobilized on a galactose-rich polysaccharide comprising

mainly galactose residues and derivatized galactose residues, collagen, gelatin, fibronectin, casein, mucin, heparan-sulfate, carrageenan, deoxyribonucleic acid, <u>or</u> adenosine triphosphate via the N-terminus region of the lactoferrin, in a native lactoferrin to isolated immobilized lactoferrin molar ratio of from about 1:1 to about 1:5 and in a concentration of from about 0.001 to about 2.5 % wt/vol.

71. The method of claim 70, wherein the composition is an aqueous solution, an aqueous emulsion, a colloid, a suspension, a powder, or a granular solid.

72. The method in accordance with claim 70, wherein the lactoferrin is immobilized on a galactose-rich polysaccharide comprising mainly galactose residues and derivatized galactose residues.

73. The method in accordance with claim 70, wherein the mixture comprises about 1 % wt/vol immobilized lactoferrin and about 1 % wt/vol native lactoferrin.

74. The method in accordance with claim 70 wherein the physiologically acceptable acid is citric acid, the physiologically acceptable base is sodium bicarbonate and the physiologically acceptable salt is sodium chloride.

75. The method of claim 70, wherein the microbe is a bacterium, a fungus, a protozoan, or a virus.

76. The method in accordance with claim 70, wherein the microbe is enterotoxigenic Escherichia coli, enteropathogenic Escherichia coli, Shigella dysenteriae, Shigella flexneri, Salmonella typhimurium, Salmonella typhi, Salmonella abony, Salmonella dublin, Salmonella enteritidis, Salmonella hartford, Salmonella kentucky, Salmonella panama, Salmonella pullorum, Salmonella rostock, Salmonella thompson, Salmonella virschow, Enterobacter aerogenes, Vibrio cholerae, Yersinia enterocolitica, Campylobacter jejuni, Aeromonas hydrophila, Staphylococcus aureus, Staphylococcus hyicus, Staphylococcus epidermidis, Staphylococcus hominis, Staphylococcus warneri, Staphylococcus xylosus, Staphylococcus chromogenes, Streptococcus pyogenes, Streptococcus pneumoniae, Streptococcus mutans, Streptococcccus sanguis, Pediococcus acne, Bacillus cereus, Bacillus anthracis, Bacillus subtilis, a Brucella species, Listeria monocytogenes, Legionella pneumophila, Bordetella pertussis, Pseudomonas aeruginosa, Francisella tularensis, Candida albicans, Brochothrix thermospacta, Bacillus pumilus, Enterococcus faecium, Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Deinococcus radiopugnans, Deinococcus radiodurans, Deinobacter grandis, Acinetobacter radioresistens, or Methylobacterium radiotolerans.

77. The method in accordance with claim 70, wherein the microbe is a verotoxic Escherichia coli.

78. The method in accordance with claim 77, wherein the verotoxic Escherichia coli is the serotype 0157: H7.

79. The method of claim 70, wherein the microbe is a Clostridium species.

80. The method of claim 79, wherein the species is Clostridium perfringens, Clostridium difficile, Clostridium botulinum, or Clostridium tetani.

81. The method in accordance with claim 70 wherein the concentration of lactoferrin on the surface of the meat product is from about 0.0001 to about 10 mg /sq. inch.

82. The method in accordance with claim 70, wherein the concentration of lactoferrin on the surface of the meat product is from about 0.01 to about 1 mg/sq. inch.

83. The method in accordance with claim 70, wherein the meat product is a beef product, a pork product, or a poultry product.

84. The method of claim 70, wherein the meat product is veal, lamb, sheep, goat, elk, deer, antelope, horse, or dog.

85. A foodstuff containing: isolated lactoferrin immobilized on a naturally occurring substrate via the N-terminus region of the lactoferrin in a concentration between about 0.0001 and about 10 mg per gram of the foodstuff.

86. The foodstuff in accordance with claim 85, wherein the composition is a meat product.

87. The foodstuff of Claim 86, wherein the meat product is a beef product, a pork product, or a poultry product.

88. The foodstuff of claim 86, wherein the meat product is veal, lamb, sheep, goat, elk, deer, antelope, horse, or dog.

89. The foodstuff of claim 86, wherein the foodstuff comprises a surface and/or flesh of a marine or freshwater aquatic organism.

90. The foodstuff of claim 89, wherein the aquatic organism is a fish, mollusk, or crustacean.

91. The foodstuff of claim 85, wherein the foodstuff comprises a vegetable foodstuff.

92. The foodstuff of claim 86, wherein said foodstuff is a packaged foodstuff.

93. A method of inhibiting the growth and/or adhesion of a microbial species on a foodstuff, comprising: treating a food-contacting surface of a material for food packaging or food handling with an isolated lactoferrin immobilized on a naturally occurring substrate via the

N-terminus region of the lactoferrin; and contacting a foodstuff with said surface, whereby the growth and/or adhesion of a microbial species on said foodstuff is inhibited.

94. The method of Claim 93, wherein said food packaging or handling material is a cellulosic polymer.

95. The method of Claim 93, wherein said food packaging or handling material is paper, wood, or cardboard.

96. The method of Claim 93, wherein said food-contacting surface comprises a surface belonging to a shear wrap, a cellophane, a wrapping paper, a waxed paper, a bag, a carton, a box, a tray, a plate, a bowl, a food storage vessel, a serving dish, a cup, a bin, a jar, or a bottle.

97. The method of Claim 97, wherein said food-contacting surface comprises a surface belonging to a glove, a mitt, a fork, a spoon, a knife, a slicer, a tong, a ladle, a scoop, a cup, a processor, a juicer, a grinder, a press, a hook, a chipper, a peeler, a cutter, a screw, an opener, a chute, a spatula, a cutting board, a kneading board, a rack, or a shelf.

98. A food container or food-handling implement, said container or implement having a food-contacting surface, said surface treated with an isolated lactoferrin immobilized on a

naturally occurring substrate via the N-terminus region of the lactoferrin in an amount effective to inhibit the growth and/or adhesion of a microbial species on said surface.

99. The food container or food-handling implement of Claim 98, wherein said container or implement is a shear wrap, a cellophane, a wrapping paper, a waxed paper, a bag, a carton, a box, a tray, a plate, a bowl, a food storage vessel, a serving dish, a cup, a bin, a jar, a bottle, a glove, a mitt, a fork, a spoon, a knife, a slicer, a tong, a ladle, a scoop, a processor, a juicer, a grinder, a press, a hook, a chipper, a screw, a cutter, a peeler, an opener, a chute, a spatula, a cutting board, a kneading board, a rack, or a shelf.

100. The food container or food-handling implement of Claim 98, having an amount of between about 0.0001 to about 10 mg /square inch of said food-contacting surface.

101. An antimicrobial cleanser, polish, paint, spray, soap, or detergent for applying to an inanimate surface, containing an isolated lactoferrin immobilized on a naturally occurring substrate not including gelatin via the N-terminus region of the lactoferrin in an amount effective to inhibit the growth and/or adhesion of a microbial species on said surface.

102. A composition of matter comprising a dispersion of isolated lactoferrin immobilized on a naturally occurring substrate not including gelatin via the N-terminus region of the lactoferrin; and at least one pharmaceutically acceptable carrier.

103. The composition in accordance with claim 102, wherein the naturally occurring substrate, not including gelatin, is a protein, a polysaccharide, cellulose, a nucleic acid, or a nucleotide.

104. The composition in accordance with claim 102, wherein the naturally occurring substrate not including, gelatin is collagen, fibronectin, casein, mucin, heparin-sulfate, carrageenan, deoxyribonucleic acid, or adenosine triphosphate.

105. The composition in accordance with claim 102, wherein the naturally occurring substrate is a galactose-rich polysaccharide comprising mainly galactose residues and derivatized galactose residues.

106. The composition of claim 105, wherein the dispersion is an aqueous solution, an aqueous emulsion, a colloid, a suspension, a powder, or a granular solid.

107. The composition in accordance with claim 102, further comprising native lactoferrin.

108. The composition in accordance with claim 107, wherein the concentration of immobilized lactoferrin and native lactoferrin in the dispersion is from about 0.05% wt/vol to about 2.5 % wt/vol.

109. The composition in accordance with claim 107, wherein the molar ratio of immobilized lactoferrin to native lactoferrin is a ratio of from about 1:1 to about 1:10.

110. The composition in accordance with claim 107, wherein the molar ratio of immobilized lactoferrin to native lactoferrin is a ratio of from about 1:1 to about 1:5.

111. The composition in accordance with claim 107, wherein the composition comprises about 1 % wt/vol immobilized lactoferrin and about 1 % wt/vol native lactoferrin.

112. The composition in accordance with claim 107, wherein the composition further comprises a buffer system.

113. The composition in accordance with claim 112, wherein the buffer system contains a physiologically acceptable acid, a physiologically acceptable base, and a physiologically acceptable salt.

114. The composition in accordance with claim 113, wherein the physiologically acceptable acid is oxalic acid, ethylenediamine tetraacetic acid, carbonic acid, or citric acid; the physiologically acceptable base is sodium bicarbonate, potassium bicarbonate, sodium carbonate, or potassium carbonate; and the physiologically acceptable salt is calcium chloride, potassium chloride or sodium chloride.

115. The composition of claim 102, wherein the carrier is selected from the group consisting of solid, semisolid or liquid glucose, lactose, sucrose, gum acacia, agar, petrolatum, lanolin, dimethyl sulfoxide, normal saline, phosphate buffered saline, sodium alginate, bentonite, carbomer, carboxymethylcellulose, carageenan, powdered cellulose, cholesterol, gelatin, hydroxyethyl cellulose, hydroxypropyl cellulose, hydroxypropyl methylcellulose, methylcellulose, octoxynol 9, oleyl alcohol, polyvinyl alcohol, povidone, propylene glycol monostearate, sodium lauryl sulfate, sorbitan esters, stearyl alcohol, tragacanth, xanthan gum, chondrus, glyercin, trolamine, avocado oil, almond oil, coconut oil, coconut butter, propylene glycol, ethyl alcohol, malt, and malt extract.

116. The composition of claim 102, further comprising a pharmaceutically acceptable emulsifier.

117. The composition of claim 116, wherein the emulsifier is selected from the group consisting of monoglyceride compounds, diglyceride compounds, glycerol, phosphatidyl ethanolamine, phosphatidyl choline, or lecithin.

118. The composition in accordance with claim 14, wherein the molar ratio of acid to base to salt is 0.01 to 0.001 M (acid): 0.1 to 0.01 M (base) : 1 to 0.1 M(salt).

119. The composition of claim 102, wherein the composition is formulated in a cosmetic, a cleanser, a food supplement, or a medicament.

120. The composition of claim 102, wherein the cosmetic, cleanser, food supplement, or medicament is formulated for applying to an external surface of a vertebrate subject.

121. The composition of claim 120, wherein the vertebrate subject is a human.

122. The composition of claim 120, wherein the vertebrate subject is a non-human vertebrate.

123. The composition of claim 119, wherein the cleanser is formulated as a pharmaceutically acceptable skin cleanser.

124. The composition of claim 119, wherein the medicament is formulated in a pharmaceutically acceptable delivery system.

125. The composition of claim 124, wherein said delivery system is an injection, intravenous drip, inhalant, or implant delivery system.

126. The composition of claim 124, wherein said delivery system is a transdermal delivery system.

127. The composition of claim 124, wherein said delivery system is a transmucosal delivery system.

128. The composition of claim 124, wherein said delivery system is an oral transmucosal delivery system.

129. The composition of claim 124, wherein said delivery system is a vaginal transmucosal delivery system.

130. The composition of claim 124, wherein said delivery system comprises an adhesive patch.

131. The composition of claim 124, wherein said delivery system comprises a gel, cream, ointment, suppository, sanitary wipe, bandage, or shampoo.

132. The composition of claim 124, wherein the delivery system is a mouth wash, gargle solution, denture cleanser, or dentifrice.

133. The composition of claim 124, wherein the delivery system is a toothpaste or chewing gum.

134. The composition of claim 124, wherein the medicament is formulated in a urogenital, rectal, or colonic delivery system.

135. The composition of claim 119, wherein the composition further comprises an antibiotic or probiotic agent.

136. The composition of claim 124, wherein the delivery system is a suppository, gel, or foam.

137. The composition of claim 127, wherein the medicament is formulated in an ingestive delivery system.

138. The composition of claim 137, wherein the ingestive delivery system is a tablet, capsule, caplet, troche, lozenge, coated or uncoated microspheres or particles, dispersible powder or granules, syrup, elixir, beverage, or food additive.

139. The composition of claim 138, wherein the tablet or capsule comprises a controlled release coating.

140. The composition of claim 138, wherein the ingestive delivery system comprises an enteric coating to prevent esophageal or gastric release of immobilized lactoferrin.

141. The composition of claim 124, wherein the delivery system comprises a lavage or enema.

142. The composition of claim 119, wherein the medicament is formulated for treating a human.

143. The composition of claim 142, wherein the composition is formulated for pediatric use.

144. The composition of claim 119, wherein the medicament is formulated for veterinary use.

145. The composition of claim 114, wherein the composition is formulated for use in a domestic or farm animal.

146. The composition of claim 114, wherein the composition is formulated for use in a non-human mammal or bird.

147. The composition of claim 146, wherein the composition is formulated for use in a non-human primate, mouse, rat, rabbit, gerbil, hamster, canine, feline, ovine, bovine, swine, pachyderm, equine, or marine mammal.

148. The composition of claim 146, wherein the composition is formulated for use in a chicken, duck, goose, turkey, ostrich, emu, dove, pigeon, quail, pheasant, peafowl, or guinea fowl.

149. The method of claim 18, wherein said composition subject to microbial contamination is a human.

150. The method of claim 149, wherein treating includes administering to said human said composition by a pharmaceutically acceptable delivery route.

151. The method of claim 150, wherein said delivery route is non-systemic.

152. The method of claim 151, wherein said non-systemic delivery route is a urogenital, rectal, or colonic delivery route.

153. The method of claim 151, wherein said non-systemic delivery route is a topical application of a cream, gel, or ointment.

154. The method of claim 150, wherein said delivery route is systemic.

155. The method of claim 154 wherein said systemic delivery route is by ingestion, injection, intravenous drip, inhalant, or implant.

156. The method of claim 154 wherein said systemic delivery route is a transdermal delivery route.

157. The method of claim 154 wherein said systemic delivery route is a transmucosal delivery route.

158. The method of claim 154, wherein the microbial contamination of a human to be reduced is in the gastrointestinal system of the human.

159. The method of claim 150, wherein treating further comprises administering an antimicrobial agent or probiotic agent in conjunction with the immobilized lactoferrin.

160. The method of claim 159, wherein the probiotic agent is a species of Bifidobacterium, Streptococcus, Pediococcus, Lactococcus, or Lactobacillus.

161. The method of claim 160, wherein the probiotic agent is Bifidobacterium bifidum, Bifidobacterium longum, Bifidobacterium animalis, Streptococcus lactis, Streptococcus cremoris, Streptococcus thermophilus, Pediococcus pentoseus, Lactococcus lactis, Lactobacillus acidophilus, Lactobacillus rhamnosus, Lactobacillus plantarum, Lactobacillus reuteri, Lactobacillus bulgaricus, Lactobacillus paracasei, or Lactobacillus casei.

162. The method of claim 159, wherein the antimicrobial agent is an antibiotic.

163. The method of claim 162, wherein the antimicrobial agent is neomycin, metronidazole, teicoplanin, vancomycin, ciprofloxacin, doxycycline, tetracycline, augmentin, erythromycin, chloramphenicol, cephalexin, penicillin, ampicillin, kanamycin, rifamycin, rifaximin, rifampin, clindamycin, trimethoprim, a 4-amino salicylate compound, a 5-aminosalicylate compound, a sulfonamide compound, a betalactam compound, an aminoglycoside compound, a macrolide compound, or a quinolone compound.

164. The method of claim 149, wherein the microbe is bacterium, a fungus, a protozoan, or a virus.

165. The method in accordance with claim 149, wherein the microbe is enterotoxigenic Escherichia coli, enteropathogenic Escherichia coli, Shigella dysenteriae, Shigella flexneri, Salmonella typhimurium, Salmonella typhi, Salmonella abony, Salmonella dublin, Salmonella enteritidis, Salmonella hartford, Salmonella kentucky, Salmonella panama, Salmonella pullorum, Salmonella rostock, Salmonella thompson, Salmonella virschow, Enterobacter aerogenes, Vibrio cholerae, Yersinia enterocolitica, Campylobacter jejuni, Aeromonas hydrophila, Staphylococcus aureus, Staphylococcus hyicus, Staphylococcus epidermidis, Staphylococcus hominis, Staphylococcus warneri, Staphylococcus xylosus, Staphylococcus chromogenes, Streptococcus pyogenes, Streptococcus pneumoniae, Streptococcus mutans, Streptococcccus sanguis, Pediococcus acne, Bacillus cereus, Bacillus anthracis, Bacillus subtilis, a Brucella species,

Listeria monocytogenes, Legionella pneumophila, Bordetella pertussis, Pseudomonas aeruginosa, Francisella tularensis, Candida albicans, Brochothrix thermospacta, Bacillus pumilus, Enterococcus faecium, Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Deinococcus radiopugnans, Deinococcus radiodurans, Deinobacter grandis, Acinetobacter radioresistens, or Methylobacterium radiotolerans.

166. The method in accordance with claim 149, wherein the microbe is a verotoxic Escherichia coli.

167. The method in accordance with claim 166, wherein the verotoxic Escherichia coli is the serotype 0157:H7.

168. The method of claim 149, wherein the microbe is a Clostridium species.

169. The method of claim 168, wherein the species is Clostridium perfringens, Clostridium difficile, Clostridium botulinum, or Clostridium tetani.

170. The method of claim 149, wherein the microbe is a protozoan selected from the group consisting of Entamoeba histolytica, Naegleria flowleri, Giardia lamblia, Leishmania spp., Trichomonas vaginalis, Trypanosoma spp., Plasmodium spp., or Taxoplasma spp.

171. The method of claim 18, wherein said composition subject to microbial contamination is a non-human vertebrate.

172. The method of claim 171, wherein treating includes administering to said non-human vertebrate said composition by a pharmaceutically acceptable delivery route.

173. The method of claim 172, wherein said delivery route is non-systemic.

174. The method of claim 173, wherein said nonsystemic delivery route is a urogenital, rectal, or colonic delivery route.

175. The method of claim 173, wherein said nonsystemic delivery route is a topical application of a cream, gel, or ointment.

176. The method of claim 172, wherein said delivery route is systemic.

177. The method of claim 176, wherein said systemic delivery route is by ingestion, injection, intravenous drip, inhalant, or implant.

178. The method of claim 176, wherein said systemic delivery route is a transdermal delivery route.

179. The method of claim 176, wherein said systemic delivery route is a transmucosal delivery route.

180. The method of claim 171, wherein the microbial contamination of a non-human vertebrate to be reduced is in the gastrointestinal system of the non-human vertebrate.

181. The method of claim 172, wherein treating further comprises administering an antimicrobial agent or probiotic agent in conjunction with the immobilized lactoferrin.

182. The method of claim 181, wherein the probiotic agent is a species of Bifidobacterium, Streptococcus, Pediococcus, Lactococcus, or Lactobacillus.

183. The method of claim 182, wherein the species is Bifidobacterium bifidum, Bifidobacterium longum, Bifidobacterium animalis, Streptococcus lactis, Streptococcus cremoris, Streptococcus thermophilus, Pediococcus pentoseus, Lactococcus lactis, Lactobacillus acidophilus, Lactobacillus rhamnosus, Lactobacillus plantarum, Lactobacillus reuteri, Lactobacillus bulgaricus, Lactobacillus paracasei, or Lactobacillus case.

184. The method of claim 181, wherein the antimicrobial agent is an antibiotic.

185. The method of claim 184, wherein the antimicrobial agent is neomycin, metronidazole, teicoplanin, vancomycin, ciprofloxacin, doxycycline, tetracycline, augmentin,

erythromycin, chloramphenicol, cephalexin, penicillin, ampicillin, kanamycin, rifamycin, rifaximin, rifampin, clindamycin, trimethoprim, a 4-amino salicylate compound, a 5-aminosalicylate compound, a sulfonamide compound, a betalactam compound, an aminoglycoside compound, a macrolide compound, or a quinolone compound.

186. The method of claim 171, wherein the microbe is a bacterium, a fungus, a protozoan, or a virus.

187. The method in accordance with claim 171, wherein the microbe is enterotoxigenic Escherichia coli, enteropathogenic Escherichia coli, Shigella dysenteriae, Shigella flexneri, Salmonella typhimurium, Salmonella typhi, Salmonella abony, Salmonella dublin, Salmonella enteritidis, Salmonella hartford, Salmonella kentucky, Salmonella panama, Salmonella pullorum, Salmonella rostock, Salmonella thompson, Salmonella virschow, Enterobacter aerogenes, Vibrio cholerae, Yersinia enterocolitica, Campylobacter jejuni, Aeromonas hydrophila, Staphylococcus aureus, Staphylococcus hyicus, Staphylococcus epidermidis, Staphylococcus hominis, Staphylococcus warneri, Staphylococcus xylosus, Staphylococcus chromogenes, Streptococcus pyogenes, Streptococcus pneumoniae, Streptococcus mutans, Streptococcccus sanguis, Pediococcus acne, Bacillus cereus, Bacillus anthracis, Bacillus subtilis, a Brucella species, Listeria monocytogenes, Bordetella pertussis, Pseudomonas aeruginosa, Legionella pneumophila, Francisella tularensis, Candida albicans, Brochothrix thermospacta, Bacillus pumilus, Enterococcus faecium, Actinobacillus actinomycetemcomitans, Porphyromonas

gingivalis, Prevotella intermedia, Deinococcus radiopugnans, Deinoeoccus radiodurans, Deinobacter grandis, Aeinetobacter radio resistens, or Methylobacterium radiotolerans.

188. The method in accordance with claim 171, wherein the microbe is a verotoxic Escherichia coli.

189. The method in accordance with claim 188, wherein the verotoxic Escherichia coli is the serotype 0157:H7.

190. The method of claim 171, wherein the microbe is a Clostridium species.

191. The method of claim 190, wherein the species is Clostridium perfringens, Clostridium difficile, Clostridium botulinum, or Clostridium tetani.

192. The method of claim 171, wherein the microbe is a protozoan selected from the group consisting of Entamoeba histolytica, Naegleria flowleri, Giardia lamblia, Leishmania spp., Trichomonas vaginalis, Trypanosoma spp., Plasmodium spp., or Taxoplasma spp.

193. The method of claim 171, wherein said non-human vertebrate is a domestic or farm animal.

194. The method of claim 171, wherein said non-human vertebrate is a mammal or bird.

195. The method of claim 171, wherein said non-human vertebrate is a non-human primate, mouse, rat, rabbit, gerbil, hamster, canine, feline, ovine, bovine, swine, pachyderm, equine, or marine mammal.

196. The method of claim 171, wherein said non-human vertebrate is a chicken, duck, goose, turkey, ostrich, emu, dove, pigeon, quail, pheasant, peafowl, or guinea fowl.

197. The method of claim 18, wherein said composition subject to microbial contamination is a biological surface or a biological fluid.

198. The method of claim 197, wherein the fluid is a culture medium.

199. The method of claim 197, wherein the biological surface or fluid is in vitro.

200. The method of claim 197, wherein the biological surface is a cell surface, membrane surface, mucosal surface, epithelial surface, lumenal surface, skin surface, or eggshell surface.

201. The method of claim 197, wherein the biological surface is an epithelial or mucosal surface.

202. The method of claim 197, wherein the biological fluid is semen, blood, lymph, urine, prostatic fluid, saliva, gastric juice, mucus, synovial fluid, pleural exudate, peritoneal exudate, pericaridal exudate, or cerebro-spinal fluid.

203. CANCELED.

(9) Evidence Appendix

The evidence appendix includes the items of evidence listed below.

1. Declaration of Dr. Andrew R. Barron dated July 26, 2004. This evidence was entered into the record as shown by the examiner's discussion of the Barron declaration on Page 9 of the examiner's Office Action mailed April 7, 2005.

2. U.S. Pat. No. 6,066,469 to Kruzel et al. This evidence was entered into the record as shown by the examiner's Notice of References Cited which was included with the examiner's Office Action dated June 30, 2003.

3. U.S. Pat. No. 6,475,511 to Gohlke et al. This evidence was entered into the record as shown by the examiner's Notice of References Cited which was included with the examiner's Office Action dated June 30, 2003.

4. WO 91/13982 to Kruzel. This evidence was entered into the record as shown by the examiner's Notice of References Cited which was included with the examiner's Office Action dated June 30, 2003.

5. EP 0 753 308 A2 to Valenti et al. This evidence was entered into the record as shown by the examiner's Notice of References Cited which was included with the examiner's Office Action dated June 30, 2003.

6. EP 0 753 309 A2 to Valenti et al. This evidence was entered into the record as shown by the examiner's Notice of References Cited which was included with the examiner's Office Action dated June 30, 2003.

7. Merriam-Webster's Collegiate Dictionary for "should." This evidence was entered into the record as shown by the examiner's discussion of the dictionary in the examiner's Advisory Action mailed September 13, 2005.

8. Concise Chemical and Technical Dictionary for "mineral oil" and "paraffin oil." The examiner's entry of this evidence is consistent with the examiner's concurrent entry of the Merriam-Webster's Collegiate Dictionary for "should."

9. Concise Chemical and Technical Dictionary for "petroleum jelly" and "Vaseline." The examiner's entry of this evidence is consistent with the examiner's concurrent entry of the Merriam-Webster's Collegiate Dictionary for "should."

DECLARATION OF DR. ANDREW R. BARRON

(37 C.F.R. § 1.132)

I, ANDREW R. BARRON, Ph.D. hereby declare and state as follows:

1. I received A.R.C.S. and B.Sc. (1st Class, Hones.) degrees, majoring in chemistry, at Imperial College of Science and Technology, University of London in 1986. I received a D.I.C. and Ph.D., specializing in inorganic chemistry at the same university in 1986. I was a Post-doctoral Research Associate, specializing in inorganic chemistry, at the University of Texas, Austin in 1986-1987.

2. I was an Assistant Professor and then an Associate Professor, specializing in inorganic chemistry, at Harvard University from 1987 to 1995.

3. In the fall of 1995, I went to Rice University, where I am currently the Charles W. Duncan, Jr. - Welch Chair of Chemistry and Professor of Materials Science in the Department of Chemistry and Department of Mechanical Engineering and Materials Science.

4. I have authored over two hundred journal articles and have made a like number of presentations.

5. I have been asked by the attorneys for the inventor Dr. A. Satyanarayan Naidu to use my technical expertise and consider an Office Action mailed January 12, 2004, in connection with the prosecution of U.S. Patent Appl. Ser. No. 09/980,062, filed on May 26, 2000 (the "Naidu application").

6. As part of my evaluation, I have studied the Naidu application and the Office Action, as well as all of the references cited in the Office Action.

<u>The Immobilization of Lactoferrin</u>

7. Lactoferrin (LF) has a bilboate structure, with a positively charged amino terminus (N-terminus) lobe and a negatively charged carbon terminus (C-terminus lobe). A full length LF peptide sequence has about 600 to about 800 continuous amino acids. Human LF in particular is about 703 amino acids long and has a molecular weight of about 83,000 Daltons.

8. The Naidu application describes LF immobilized on a naturally occurring substrate via its N-terminus region, *i.e.,* LF having its N-terminus region attached to a substrate leaving the C-terminus region free. (Naidu Appl. page 7, line 34-page 8, line 3.)

9. For the N-terminus region to become immobilized on a naturally occurring substrate, the region of the substrate to which the N-terminus region is to become attached should carry the opposite charge, *i.e.*, carry a negative charge.

US 6,475,511 B2

10. I have studied US 6,475,511 B2 by Gohlke and Cockrum ("Gohlke *et al.*"). Gohlke *et al.* describe compositions containing a combination of colostrum and lactoferrin in a "mucosal delivery format" ("MDF"). (Col. 6, lines 13-28.) The composition can also contain modified pectin. (Col. 6, lines 49-52.)

11. By MDF is meant a composition, such as a lozenge, formed of solid components. For example, Gohlke *et al.* teach, "The individual components of the composition may be obtained from commercial sources: colostrum (which is dehydrated by standard spray-drying procedures known in the art)" (col. 9, lines 41-44). Furthermore, examples 1 - 3 describe a process for preparing the product where, "[E]ach of the following ingredients is placed, in powdered form, into a commercial mixer." The ingredients are then mixed and cold pressed.

12. I have considered the examiner's suggestion that:

> "Gohlke *et al.* teach lactoferrin combined with colostrum . . . , pectin, and pharmaceutically acceptable carriers such as dextrose (see, *e.g.*, Examples 1-3). The components are thoroughly mixed and cold pressed to form a lozenge . . . Because the same components are present in the same compositions, inherently the lactoferrin in the lozenges of Gohlke *et al.* will be immobilized via its N-terminus to the proteins, polysaccharides, and lipids which are present to the same extent claimed by Applicant."

13. The mere presence of LF in a cold-pressed mixture with other solids, such as colostrum and modified pectin in an MDF format, would not inherently result in the LF becoming attached via its N-terminus.

14. Gohlke *et al.* do not disclose nor suggest any conditions under which the compounds could be mixed to result in the LF becoming attached via its N-terminus. For example, mixing LF with colostrum (and modified pectin) and cold pressing will not provide an

environment suitable to cause the LF to become attached to colostrums or modified pectin via LF's N-terminus region.

WO Patent Application 91/13982

15. I have studied WO Patent Application 91/13982 ("WO Patent Application '982"). This reference generally relates to human LF expressed using recombinant DNA. It discloses the use of this LF as a nutritional supplement, an antiseptic, and as a food-spoilage retardant. The LF can be compounded with certain carriers or diluents. It neither broadly teaches LF immobilized on a naturally occurring substrate via the N-terminus region of the LF, nor does it provide a specific example of such an immobilized LF.

16. I have considered the examiner's suggestion that:

> "The WO Patent Application '982 teaches LF in combination with stearic acid (which is a lipid and also corresponds to Applicant's pharmaceutically acceptable carrier of claim 102) or its salts . . . Because the same components are present in the same defined dispersion; inherently the LF in the composition of the WO Patent Application '982 will be immobilized via its N-terminus."

17. Stearic acid with a molecular weight of only 284.47 is not a substrate. LF could not become immobilized on such a small molecule. That would be akin to saying that a dog was immobilized on a flea, if a flea attached itself to a dog.

18. WO Patent Application '982 does not disclose nor suggest any other carriers or diluents that would be reactive with the N-terminus of LF and immobilize LF.

19. Furthermore, the mere presence in a mixture of LF and stearic acid or any of the other naturally occurring carriers or diluents taught in WO Patent Application '982 would not inherently result in the LF becoming attached via its N-terminus on a substrate.

20. WO Patent Application '982 does not disclose nor suggest any conditions under which the compounds could be mixed to result in the LF becoming attached via its N-terminus. Merely compounding solid LF with other solids, such as stearic acid, will not provide an

environment suitable to cause the LF to become attached to the other solid via LF's N-terminus region.

European Patent Application 753,309

21. I have studied European Patent Application 753,309 (European Patent Application '309). This reference generally relates to the preparation of mixtures of LF and desferrioxamine methanesulphonate useful for the therapy of viral infectious diseases. It neither broadly teaches LF immobilized on a naturally occurring substrate via the N-terminus region of the LF, nor does it provide a specific example of such an immobilized LF.

22. I have considered the examiner's suggestion that:

> "The European Patent Application '309 teaches compositions comprising LF and carriers such as paraffin oil and Vaseline (which are lipids), xantan gum and corn starch (which are polysaccharides), and lecithin (which is an emulsifier) . . . Because the same components are present in the same defined dispersion, inherently the LF in the composition of the European Patent Application '309 will be immobilized by its N-terminus . . ."

23. Paraffin oil and Vaseline are low molecular weight compounds, not substrates. LF could not become immobilized on such small molecules.

24. Paraffin oil and Vaseline do not have any reactive substituents or groups that would react with the N-terminus of LF. LF could not become immobilized on such inert molecules.

25. Furthermore, paraffin oil and Vaseline are hydrocarbons (not lipids) and do not carry any charge. As a result, neither paraffin oil nor Vaseline contains a region that will attach LF's positively charged N-terminus region.

26. Xantan gum and cornstarch do not carry any charges. As a result, neither xantan gum nor cornstarch contains a region that will attach LF's positively charged N-terminus region.

27. Lecithin is a low molecular weight compound. LF could not become immobilized on such a small molecule.

European Patent Application 753,308

28. I have studied European Patent Application 753,308 (European Patent Application '308). European Patent Application '308 generally relates to the use of LF for therapy of diseases caused by Gram-positive pathogen microorganisms. It neither broadly teaches LF immobilized on a naturally occurring substrate via the N-terminus region of the LF, nor does it provide a specific example of such an immobilized LF.

29. I have considered the examiner's suggestion that:

> "The European Patent Application '308 teaches compositions comprising LF and peppermint oil, gum base and corn starch (which are polysaccharides) . . . Because the same components are present in the same defined dispersion, inherently the LF in the composition of the European Patent Application '308 will be immobilized via its N-terminus . . ."

30. Peppermint oil is a low molecular weight compound, not a substrate. LF could not become immobilized on such a small molecule.

31. Peppermint oil, gum base and cornstarch do not carry any charges. As a result, neither gum base nor cornstarch contains a region that will attach LF's positively charged N-terminus region.

US Patent 6,066,469

32. I have studied US Patent 6,066,469 by Kruzel *et al.* ("Kruzel *et al.*"). This reference generally relates to LF expressed using recombinant DNA. It discloses the use of this LF as a nutritional supplement, an antiseptic, to treat and prevent opportunistic bacterial, viral and fungal infections, and as a food-spoilage retardant. It neither broadly teaches LF immobilized on a naturally occurring substrate via the N-terminus region of the LF, nor does it provide a specific example of such an immobilized LF.

33. I have considered the examiner's suggestion that:

> "Kruzel *et al.* teach nutritional supplements comprising LF in combination with adjuvants or diluents such as cellulose, starch,

tragacanth, and sodium carboxymethlycellulose . . . Because the same components are present in the same defined dispersion, inherently the LF in the nutritional supplements of Kruzel *et al* will be immobilized via its N-terminus . . ."

34. The mere presence in a mixture of LF and an adjuvant or a diluent, such as the solids cellulose, starch, tragacanth, and sodium carboxymethlycellulose would not inherently result in the LF becoming attached via its N-terminus.

35. Kruzel *et al.* does not disclose nor suggest any conditions under which the compounds could be mixed to result in the LF becoming attached via its N-terminus. Merely compounding solid LF with a solid adjuvant or a a diluent, such as cellulose, starch, tragacanth, and sodium carboxymethlycellulose will not provide an environment suitable to cause the LF to become attached to stearic acid via LF's N-terminus region.

36. Cellulose and starch do not carry any charges. As a result, neither cellulose nor starch contains a region that will attach LF's positively charged N-terminus region.

37. Furthermore, the mere presence in a mixture of LF and any of the adjuvants or diluents, such the solids cellulose, starch, tragacanth, or sodium carboxymethlycellulose would not inherently result in immobilization of the LF via its N-terminus.

38. Merely compounding LF with other solids, such as by cold-pressing two or more solids, will not provide an environment suitable to cause the LF to become immobilized via its N-terminus region.

I hereby declare that all of the statements herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

I declare under the penalty of perjury, under the laws of the State of ___Texas___, that the foregoing is true and correct.

Dated: July __26__, 2004

____
Dr. Andrew R. Barron

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(54) Title: GENETICALLY ENGINEERED HUMAN LACTOFERRIN

(57) Abstract

Disclosed is human lactoferrin expressed from recombinant DNA, its method of production and purification and its use.

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INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/01335

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12N 15/12,15/63; A23L,3/3463 ; A01N 37/18

U.S.CL.: 435/69.1,240.1,252.3; 536/27; 99/485; 514/2

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
US	435/69.1,240.1,252.3; 536/27; 99/485; 514/2

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

CA: KEY WORDS: LACTOFERRIN, DISINFECTANT, NUTRITIONAL SUPPLEMENT, FOOD SPOILAGE RETARDANT

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [*]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	US,A. 4,436,658 (PEYROUSET ET AL) 13 March 1984. see entire document.	1-7.16-29
Y	BLOOD, Vol. 70, No. 4 issued October 1987, 1987, Rado et al. "Isolation of Lactoferrin cDNA From a Human Myeloid Library and Expresion of mRNA During Normal and Leukemic Myelopoiesis", pages 989-993, see entire document.	8-15,38-39 17,19,22, 24,27,29
Y	BIOCHEMISTRY Vol. 28, NO. 9, issued 1989, SREEKRISHNA ET AL. "High-Level Expression, Purification, and Characterization of Recombinant Human Tumor Necrosis Factor Synthesized in the Methylotrophic Yeast *Pichia pastoris*", pp 4117-4125, see entire document.	10

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
21 MAY 1991	26 JUL 1991
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Sharon L. Nolan Sharon L. Nolan

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	TIBTECH, Vol. 5 issued August 1987, Porath et al "Thiophilic interaction and the selective adsorption of proteins", pp. 225-229, see entire document.	12-15
Y	Sulkowski, "Protein Purification: Micro to Macro" published 1987 (Department of Moclecular and Cellular Biology, Roswell Park Memorial Institute, Buffalo, N.Y), pp. 177-195, see entire document.	12-15
Y	Clinica Chimica Acta, Vol. 157, issued 1986, Bezwoda et al, "Isolation and Characteriasation of lactoferrin separated from human whey by adsorption chromatography using Cibacron Blue F3G-A linked affinity adsorbent", pages 89-93, see entire document.	12-15 30-37

Form PCT/ISA/210 (extra sheet) (Rev.11-87)

International Application No. PCT/US91/01335

FURTHER INFORMAT CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers , because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. ☐ Claim numbers__________, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1) Product (cl. 1-7), Method of making recombinantly (cl.8-15 & 38-39), + Method of use for inhibiting microbial growth (cl. 16-17 & 25-27). II) Method of making by purification (cl. 30-37) III) Method of use as nutritional supplement (cl. 18-20, 23-24) IV)Method of use as food spoilage retardant (cl. 21-22, 28-29).

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application. Telephone practice
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (supplemental sheet (2) (Rev. 11-87)

GENETICALLY ENGINEERED HUMAN LACTOFERRIN

The present invention relates to human lactoferrin. In particular, it relates to human lactoferrin, its production, and its use.

Lactoferrin plays an important role in iron transport and utilization in humans. A glycoprotein containing about 6% sugar and having a total molecular weight of about 80,000 daltons, human lactoferrin is capable of binding two ferric ions with high affinity. The binding constant of iron by lactoferrin is about $10^{20}\ M^{-1}$. Although lactoferrin binds iron tightly, the binding is reversible so that the metal is available upon demand to cells with a need for this essential element.

Human milk is high in lactoferrin content. The high degree of iron absorption from human milk is manifested in a low incidence of iron deficiency anemia among breast fed infants compared to infants fed with cow's milk. Accordingly, lactoferrin is a key protein for healthy development of infants. However, production of lactoferrin from human milk poses problems. First, the severely limited amount of human milk, the major source of human lactoferrin, that is available restricts lactoferrin production. Secondly, production of lactoferrin from human milk presents a tremendous risk factor of infectious contamination. That is, it could carry with it a potentially lethal contaminant, such as the AIDS virus, or another undesirable agent.

Accordingly, the present invention provides human lactoferrin expressed from recombinant DNA. The lactoferrin of the present invention is free of naturally occurring contaminants, e.g., proteins and viruses, that would be detrimental to the recipient. The present invention also provides a genetically altered organism capable of producing human lactoferrin. In a further aspect, the present invention provides a method of producing human lactoferrin comprising the steps of (a) isolating DNA encoding human

lactoferrin from a cDNA library derived from human breast tissue;

(b) inserting the isolated DNA into the DNA of a host organism; (c) culturing the host organism to express human lactoferrin; and (d) recovering the expressed lactoferrin from the culture media. In still another aspect of the present invention there is provided a process for inhibiting microbial growth in a mammal comprising topically applying to the subject a therapeutically effective amount of lactoferrin having less than 25% metal loading, a process for inhibiting a trace-element deficiency in a mammal comprising orally administering a therapeutically effective amount of lactoferrin having at least 35% trace-element loading. The present invention also provides a nutritional supplement comprising the trace-element loaded human lactoferrin having at least 35% metal loading and a nutritionally acceptable carrier or diluent. The present invention further provides a topical antiseptic comprising an effective amount of lactoferrin having less than 25% metal loading and a pharmaceutically acceptable carrier or diluent. Still another aspect of the present invention provides a process for inhibiting food spoilage comprising adding to the food an effective amount of lactoferrin having less than 25% metal loading.

The present invention also contemplates an improvement in a chromatography process useful in purifying lactoferrin and other proteins comprising the steps of (a) contacting a substance with a first adsorbent to obtain adsorbed and non-adsorbed fractions, (b) eluting the adsorbed fraction with an eluant and (c) contacting the adsorbed fraction with a second adsorbent, wherein the improvement comprises equilibrating the second adsorbent with the eluant followed

by contacting the eluate containing the adsorbed fraction with the second adsorbent.

Fig. 1 is a schematic diagram showing a preferred embodiment of the improved chromatography method of the present invention. Fig. 2 is a flow chart showing a preferred embodiment of purifying lactoferrin in accordance with the present invention.

Lactoferrin is produced according to the present invention using recombinant DNA technology. That is, by using recombinant DNA technology, a polypeptide containing the primary structural conformation of naturally occurring human lactoferrin and possessing its biological properties is produced. The preferred source of DNA encoding lactoferrin is a cDNA library derived from human RNA and ligated to an appropriate vector according to methods that will be readily apparent to the skilled artisan, e.g., as disclosed in Davis, et al., <u>Basic Methods in Molecular Biology</u>, Elsevier Science Publishing Co., Inc, (1986), the disclosure of which is incorporated herein by reference. In a preferred embodiment, the RNA is isolated from the human mammary gland and the vector is phage λ gt 11. The cDNA library is screened for positive (DNA carrying the lactoferrin gene) clones using techniques that will be readily apparent to the skilled artisan, such as disclosed in the aforesaid Davis, et al., publication and Rado, et al., <u>Blood, 70</u>, No. 4 (October, 1987) pp. 989-983, the disclosure of which is incorporated herein by reference. For example, the cDNA is hybridized to a radiolabeled oligonucleotide probe and the positive clones identified by autoradiography. Preferably, positive clones are identified using lactoferrin antisera, and the antisera-containing clones are recognized using an appropriate development system, such as an avidin/biotin reaction system. Large numbers of positive clones are then generated by

infecting an appropriate bacterial host, such as E. coli Y 1090, using methods that will be readily apparent to the skilled artisan such as disclosed in the aforesaid Davis, et al., publication. DNA is then isolated from the clones. The cDNA encoding lactoferrin is then cut with an appropriate restriction endonuclease such as EcoRI. The cut DNA encoding lactoferrin is separated by chromatography. Preferably, the separated cDNA is further sub-cloned into another vector, such as the pGEM-4 plasmid, and the inserted cDNA again excised and separated.

Expression of human lactoferrin according to the present invention is performed using an appropriate expression vector, such as the plasmid pAO804, and an appropriate host organism, such as the yeast Pichia pastoris. Other useful hosts include a mammalian cell line such as Chinese hamster ovary (CHO) and expression vectors that will be readily apparent to the skilled artisan. Insertion of the cDNA and expression of the human lactoferrin are carried out according to techniques that will be readily apparent to the skilled artisan, such as disclosed in Rothstein, Methods in Enzymology, 101, 202-210 (1983), and Tschopp, et al., Bio/Technology, 5, 1305-1308 (1987), the disclosures of which are incorporated herein by reference.

Purification of the expressed protein according to the present invention is preferably carried out by one of several methods. In one preferred embodiment, cell-free culture media containing the expressed lactoferrin is passed through a filter that retains material having a molecular weight greater than about 10,000 daltons and then sterilizing the retained protein. The material retained by the filter is subjected to a two-step affinity chromatography process. In the first step, the affinity ligand is the reactive dye Cibacron blue F3G-A (color index (C.I.) 61211, λ max

605(374)nm) disclosed in Bezwoda, et al., Clin. Chim. Acta., 157, 89-94 (1986), and Chemical Abstracts Service (CAS) No. 12236-82-7, the disclosures of which are incorporated herein by reference. Cibacron blue F3G-A can be covalently bound to a cross-linked agarose gel by the triazine coupling method as described in Bohme, et al., J. Chromatography, 69, 209-214 (1972), the disclosure of which is incorporated herein by reference. In the second step, controlled-pore glass (CPG) or silicic acid is used to further purify the adsorbed material obtained in the first step. In another preferred embodiment, the adsorbed material from the CPG or silicic acid is further chromatographed in a third step using one of the following chromatography techniques before final filtration and sterilization: T-Gel chromatography; immobilized metal-ion affinity chromatography (IMAC) using a metal ion capable of forming a complex with lactoferrin, such as nickel, which can be coupled with an imminodiacetic acid-epoxy activated gel (IDA Me(II)) in accordance with Sulkowski, Frontiers in Bioprocessing, Sidkar et al., ed., 343-353 (1990), the disclosure of which is incorporated herein by reference; or chromatography with the ligand phenyl glycidyl ether, which can be coupled to a cross-linked agarose gel as disclosed in Janson and Riden, Protein Purification Principles High Resolution Methods and Applications, VSH Publishers New York (1989), incorporated by reference herein. The two-step and three-step methods are schematically presented by the flow chart in Fig. 2.

The improved chromatography process of the present invention is useful in purifying proteins, such as lactoferrin produced in accordance with the present invention. As shown in Fig. 1, crude fermentation broth contained in tank **1** passes to permeable membrane **3**, which retains material having a molecular weight greater than

10,000 daltons and passes an ultrafiltrate containing water, salts, and low-molecular-weight proteins. The retained material is washed with a buffer and further concentrated. The washed material is then applied to chromatography column 5 containing an adsorbent that has been equilibrated with the buffer used to wash the filtered material while valve 7 is open and valve 9 is closed. After non-adsorbed material is discharged through valve 7, valve 7 is closed and valve 9 opened. Adsorbed material is then eluted, and the eluate passed directly to the second column 11, containing an adsorbent previously equilibrated with the eluant used to elute the adsorbed material. Use of the same medium to elute material from the adsorbent in column 5 and equilibrate the adsorbent column 11 avoids the need for timely and involved medium exchange procedures between the two adsorption steps. Passage of the adsorbed material through column 11 occurs while valve 13 is open and valve 15 is closed. Eluting adsorbed material from column 11 occurs while valve 13 is closed and valve 15 is open, thereby passing eluate from column 11 directly to a filter (not shown) capable of retaining material having a molecular weight of at least 10,000 daltons. Although demonstrated for use in purifying lactoferrin, the aforesaid method and apparatus is contemplated in other tandem chromatography procedures that will be readily apparent to the skilled artisan. For example, the invention is useful in purifying proteins with similar hydrophobicity to lactoferrin.

The nutritional supplement of the present invention contains an effective amount of lactoferrin loaded with one or more trace elements, either alone or in combination with one or more nutritionally acceptable carriers or diluents. Preferred nutritional supplements include tablets, gelatin capsules, or liquids containing the lactoferrin together with

diluents, such as lactose, dextrose, sucrose, mannitol, sorbitol, cellulose, and glycine; binders, such as magnesium aluminum silicate, starch paste, gelatin, tragacanth, methylcellulose, sodium carboxymethylcellulose, and polyvinylpyrrolidone; disintegrants, such as starches, agar, alginic acid or its sodium salt, and effervescent mixtures; as well as absorbents, colorants, flavors and sweeteners. Alternatively, the trace-element loaded lactoferrin can be added to foods such as baby formula, cereal, and ice cream to enhance the nutritional value of the food. The preferred amount of trace element-loaded lactoferrin in the supplement based on the weight of 1g of the supplement is 5-50 mg, more preferably 20-30 mg, most preferably 25 mg. An effective daily amount of trace element-loaded lactoferrin varies, based on the individual, from about 10-30 mg, preferably 20-30 mg, more preferably 25 mg. The preferred trace element in the supplement is iron, but other trace elements, e.g., zinc and copper, are contemplated within the scope of the present invention. Loading lactoferrin with the appropriate trace element is accomplished by simple titration with, e.g., ferrous ammonium in the presence of bicarbonate, according to methods that will be readily apparent to the skilled artisan. Preferred loading is such that at least 35%, more preferably at least 50%, most preferably at least 70%, of the metal-binding sites are bound to the trace element. The lactoferrin contemplated for use in the supplement is preferably of human derivation, more preferably via DNA recombinant means, but other lactoferrins, such as bovine and porcine lactoferrin, are contemplated.

Lactoferrin can be used as an antiseptic in accordance with the present invention either alone or in the form of a powder, solution, ointment, aerosol spray, or cream to any part of the subject as an aid in the prevention or treatment

of microbial infections. By depriving the surrounding environment of iron, lactoferrin inhibits the growth microbes, such as bacteria. Preferable antiseptics of the present invention include lactoferrin either alone or compounded with carriers such as, saline silica, talcum, stearic acid, its magnesium or calcium salt, polyethyleneglycol, and fatty emulsions and suspensions that will be readily apparent to the skilled artisan. The lactoferrin is preferably present in the antiseptic based on 1 ml of the carrier at 0.1-2 mg, preferably 0.2-2 mg. An effective amount of the lactoferrin varies depending on the individual treated, severity of infection, if any, and the area to which administration is contemplated. Preferably, in treating mammals a twice-daily administration of 0.1-2 mg, more preferably 0.5-2 mg, most preferably 1 mg, of lactoferrin per 0.1 square meter effected area is contemplated. For use as an antiseptic in accordance with the present invention the lactoferrin preferably has less than 25% of its metal-binding sites loaded, i.e., at least 75% of its metal-binding sites are available to sequester iron. More preferably, less than 20% of the metal-binding sites are loaded, most preferably less than 10%.

Lactoferrin can be applied to food (either solid or liquid) to retard spoilage in accordance with the present invention either alone or compounded with any of the aforesaid nutritionally acceptable carriers or diluents. By sequestering iron, and thereby suppressing its catalytic activity, the lactoferrin reduces the iron available for either microbial multiplication or the production of potentially cell-damaging free-radicals that are formed in iron catalyzed reactions from hydrogen peroxide or superoxide. For example, the lactoferrin is particularly useful in inhibiting rancidity in meat, which is iron-

dependent lipid peroxidation. To inhibit microbial growth, particularly in liquid foods such as beer and wine, the lactoferrin can be added directly to the liquid or used to coat filters through which the liquid food passes during processing. An effective amount of the lactoferrin for retarding spoilage varies depending on the type and amount of food contemplated. Preferably, the amount of lactoferrin applied to food in accordance with the present invention varies from 0.1-1 mg/ml of food with which it is mixed, or based on the surface area of the filter or solid food to which it is applied from 0.1-1 mg/6 cm^2. The preferred amount of lactoferrin compounded with a carrier in a food additive for retarding spoilage varies based on 1 ml of the carrier from 0.1-2 mg, preferably 0.2-2 mg. The same amounts of metal loading preferred for the antiseptic of the present invention are preferred for the food-spoilage retardant.

The antiseptic, dietary supplement, and food-spoilage retardant of the present invention can be sterilized and/or contain adjuvants, such as preserving, stabilizing, wetting or emulsifying agents, solution promoters, salts for regulating osmotic pressure and/or buffers. In addition, they may also contain other therapeutically valuable substances. Said compositions are prepared according to conventional mixing, granulating, and coating methods.

To more clearly describe the present invention, the following non-limiting examples are provided. In the examples all parts and percentages are by weight unless indicated otherwise.

EXAMPLE 1

In this example, human lactoferrin is obtained from a genetically altered organism. Using breast tissue excised during the mastectomy of a woman during the eighth month of pregnancy, a human mammary gland genomic library (cDNA

ligated to λ gt 11) is prepared according to Gubler, et al., Gen., 40, 1-8 (1983) (available from Clontech, California). The library is transferred onto agar plates containing a high density of E. coli Y 1090 (available from Clontech, California) (5 x 10^4 plaques per 90 mm plate or 1.4 x 10^5 plaques per 150 mm plate. The plates are allowed to stand for 3.5 hours at 42°C to obtain a lytic growth of the phage. The plates are then overlaid with nitrocellulose filters (available from Schleiher and Schnell Inc. Wouburn, MA, under no. BA 85 NC) and heated in an incubator at 37°C for 3.5 hours.

Positive clones (i.e., containing the cDNA) are identified on the membranes using rabbit antibody to natural human lactoferrin purified in accordance with Example 8 herein. Nitrocellulose filters are removed from the plates after plaque transferral, and the coated with the antibody purified in accordance with Example 8, which hybridizes with positive plaques. Following removal of excess antibody, positive plaques are developed by first applying an anti-rabbit IgG conjugated with biotin (available from Sigma Chemical Co., St. Louis, MO), and then, following removal of excess biotin conjugate, applying avidin conjugated with horse radish peroxidase (available from Sigma Chemical Co., St Louis, MO). Finally, the positive plaques are identified in the reaction catalyzed by horse radish peroxidase using as an enzyme substrate 4-chloro-1-Naphtol.

The positive plaques are then used to infect E. Coli Y 1090 to produce large amounts of phage in accordance with procedures set forth in Davis, et. al., supra. The resulting bacteriophage is purified using 10% polyethylene glycol and DNA is isolated from the phage according to the procedures disclosed in Kislow, N.A.R., 14, 6767 (1986), the disclosure of which is incorporated herein by reference. Following the

procedures in Davis, et al., supra, the cDNA insert encoding lactoferrin is sub-cloned as follows: the cDNA insert is cut out from the phage DNA using EcoRI and purified using a high resolution ion-exchange chromatography column (Gen-Pak™ Fax available from Millipore Corporation, Waters Chromatography Division, Milford, MA). The thus purified cDNA insert is ligated using T4 DNA ligase into plasmid pGEM-4 (available from Promega, Madison, WI, and described in Yanish-Perron et al. (1985), GEN, 33, 103-109) that has been cut using EcoRI using standard techniques. The plasmid containing the insert is then transferred into *E. coli* JM109 (available from Promega, Madison, WI, and described in Hanahan, J. Mil. Biol., 166, 557 (1983)). The bacteria are transferred to agar plates containing ampicillin and the positive colonies grown. The plasmid is then isolated and the cDNA insert is cut from the plasmid using EcoRI and purified by ion-exchange chromatography as described above.

The cDNA insert is then ligated into the *Pichia pastoris* expression vector pAO804 (using the *P. pastoris* GTS 115 strain) so as to be flanked by the 5' and 3' regulatory sequences of the methanol-induced alcohol oxidase gene (AOX1) of *P. pastoris* in accordance with the procedures described in Sreekrishna, et al., Biochemistry, 28, 4117-4125 (1989), and Rothstein, Methods in Enzymology, 101, 202-210 (1983), the disclosures of which are incorporated herein by reference. The thus modified vector is then grown in minimal media as described in Creeg, et al., Mol. Cell. Biol., 5, 3376-3385 (1985), the disclosure of which is incorporated herein by reference. Following the procedure of Hagenson, et al., Enzyme Microb. Technol, 11, 650-656 (1989), the cells are grown to an OD_{600} of about 1.0 and then harvested, and washed with and suspended in minimal methanol media at an OD_{600} of about 4.0. The culture is held at 30°C while

maintained at a pH of 5.0 by adding NH_3 gas to the air stream. Expressed lactoferrin is recovered from the supernatant of the fermentation media following 15' centrifugation at 5000 rpm using a Beckman J-21B with a Rotor JA 14.

EXAMPLE 2

In this example, human lactoferrin is purified in accordance with the present invention. One liter of the supernatant from Example 1 is adjusted to about 4°C and filtered under pressure through a polysulfone ultrafiltration membrane having a pH operating range of 1-14 on a polypropylene mesh support (Pellicon™ Cassette filter System assembled with Procon pump and PTGC membrane available from Millipore) to retain proteins in excess of about 10,000 molecular weight. Pressure with simultaneous circulation is applied until 900 ml of ultrafiltrate is collected. A flow rate of about 100 ml per minute is maintained during the filtration process. The retained material (100 ml) is diluted with 900 ml 20 mM phosphate buffer (pH 7.4) and re-filtered, which is repeated four times (final exchange ratio = 10,000). The final material retained is sterilized (0.22 μm Gelman™ filter).

EXAMPLE 3

In this example human lactoferrin is purified in accordance with a preferred embodiment of the present invention using affinity chromatography in which the affinity ligand is the reactive dye Cibacron blue F3G-A. The sterilized material obtained in Example 2 is adjusted to a pH of 7.5 and a final concentration of sodium chloride of 0.5 M. This material is then applied onto a column (5 cm x 35 cm) packed with cross-linked agarose coupled to the dye (available from Pharmacia Fine Chemicals, Upsala, Sweden, under the name Blue Sepharose™ CL-6B) and previously equilibrated with 50 mM

N-[2-hydroxyethyl]-piperazine-N'-[2-ethanesulfonic acid] (HEPES) buffer (pH 7.5) containing 0.5 M sodium chloride. Adsorption is performed at a flow rate of 1 ml/min followed by washing the column with 2 bed volumes of the same HEPES buffer. The non-adsorbed fraction is discarded and the adsorbed fraction containing lactoferrin is eluted from the column bed using 2 bed volumes of 50 mM HEPES buffer (pH 7.5) containing 1 M sodium chloride.

EXAMPLE 4

In this example human lactoferrin is purified in accordance with a preferred embodiment of the present invention using control pore glass (CPG) chromatography. The eluate from Example 3 is applied onto a column (1.2 cm x 10 cm) packed with CPG beads (CPG 00350 available from Electro-Nucleonics, Fairfield, N.J.) and previously equilibrated with 50 mM HEPES buffer (pH 7.5) containing 1 M sodium chloride. Adsorption is performed at a flow rate of 1 ml/min followed by washing the column with 2 bed volumes of the same buffer. The non-adsorbed fraction is discarded, and the adsorbed fraction is eluted with 2 bed volumes of 0.25 M tetramethylammonium chloride (TMAC; pH 7.5). The eluate is filtered on a membrane capable of excluding material having a molecular weight greater than 10,000 daltons (Amicon™ YM 10). The filtered material is then sterilized (0.22 μm Gelman™ filter) and frozen at -20°C.

EXAMPLE 5

In this example human lactoferrin is purified in accordance with a preferred embodiment of the present invention using immobilized metal ion affinity chromatography (IMAC). An imminodiacetic acid-epoxy activated gel (available from Pharmacia Fine Chemicals, Upsala, Sweden, under the name Chelating Sepharose™ 6B) is washed with water and equilibrated with 0.1 M sodium acetate buffer (pH 4.0)

containing 1 M sodium chloride. The gel is then packed into a chromatographic column (1.2 cm x 10 cm) and saturated with 4 bed volumes of the same sodium acetate buffer further containing 5 mg/ml of nickel chloride. Excess metal is washed from the column with the sodium acetate buffer, and the gel is equilibrated with 20 mM HEPES buffer (pH 7.0) containing 1 M sodium chloride and 2 mM imidazol.

The product of Example 4 is mixed with HEPES, sodium chloride, and imidazol to obtain a pH of 7.0, 20 mM HEPES, 1 M sodium chloride, and 2 mM imidazol. The mixture is applied onto the column at a flow rate of about 1 ml/min followed by washing the gel with 2 bed volumes of 20 mM HEPES buffer (pH 7.0) containing 1 M sodium chloride and 2 mM imidazol. The non-adsorbed fraction is discarded, and the adsorbed fraction containing lactoferrin is eluted using 2 bed volumes of 20 mM HEPES buffer (pH 7.0) containing 1 M sodium chloride and 20 mM imidazol.

EXAMPLE 6

In this example human lactoferrin is purified in accordance with a preferred embodiment of the present invention using T-Gel affinity chromatography. T-gel adsorbent is prepared according to Porath, et al., <u>Methods in Enzymology, 44</u>, 19-45 (1976), the disclosure of which is incorporated herein by reference, and packed into a column (1.2 cm x 10 cm). The final product of Example 4 is adjusted to a pH of 7.5 and a final concentration as follows: 50 mM PIPES buffer (piperazine-N,N'-bis[2-ethanesulfonic acid] and 1,4-piperazinediethanesulfonic acid] buffer and 0.7 M ammonium sulfate. The adjusted material is applied on the column that has been previously equilibrated to 50 mM PIPES buffer (pH 7.5) containing 0.7 M ammonium sulfate with a flow rate of about 1ml/min. The non-adsorbed fraction containing lactoferrin is adjusted to a concentration of 0.1 M ammonium

sulfate and then applied to an identical T-gel column previously equilibrated to 50 mM PIPES buffer (pH 7.5) containing 1.0 M ammonium sulfate. The column is then washed with 7-8 bed volumes of 50 mM PIPES buffer (pH 7.5) containing 1.0 M ammonium sulfate, with lactoferrin being present in the non-adsorbed fraction.

EXAMPLE 7

In this example human lactoferrin is purified in accordance with a preferred embodiment of the present invention using a hydrophobic interaction chromatography on a cross-linked agarose gel coupled to phenyl glycidyl ether (Phenyl Sepharose™ CL-4B available from Pharmacia Fine Chemicals, Upsala, Sweden). The gel is packed into a column and equilibrated to 50 mM PIPES buffer (pH 7.0) containing 1 M ammonium sulfate. The product of Example 4 is adjusted to the equilibrating buffer and applied onto the column at a flow rate of 1 ml/min. The non-adsorbed fraction is discarded and the adsorbed fraction containing lactoferrin is eluted using 2 bed volumes of 50 mM PIPES buffer (pH 7.0).

EXAMPLE 8

In this example anti-lactoferrin serum is purified by affinity chromatography for use in accordance with a preferred embodiment of the present invention as described in Example 1 herein. The adsorbent substrate for affinity chromatography is prepared by cyanogen bromide activation as described by Axen et al., Nature, 214, 1302-1304 (1967). The substrate (available from Pharmacia Fine Chemicals, Upsala, Sweden, under the name CNBr-Sepharose™-4B) is coupled to human lactoferrin, which acts as the affinity ligand, as follows. One gram of substrate is swelled with 1 mM HCl and washed with the same solvent on a sintered glass filter. Ten mg of natural human lactoferrin (available from Sigma Chemical Co., St. Louis, MO) is dissolved in 0.1 M $NaHCO_3$

buffer (pH 8.3) containing 0.5 M sodium chloride (coupling buffer). The resulting solution is mixed with the washed substrate gel for 2 hours, and then mixed with 0.2 M glycine buffer (pH 4.0) for 2 hours. The gel is then washed with coupling buffer, followed by 0.1 M acetate buffer (pH 4.0) containing 0.5 M sodium chloride, followed again by coupling buffer to form the adsorbent. The adsorbent is packed into a column and washed with 20 mM phosphate buffer (pH 7.4) containing 0.5 M sodium chloride. Anti-lactoferrin serum obtained from an inoculated rabbit (available from Sigma Chemical Co., St Louis, MO) is passed through the column at a flow rate of 1 ml/min and the non-adsorbed material discarded. Adsorbed material containing the purified protein is eluted with 2 bed volumes of 0.2 M glycine buffer (pH 2.0) containing 0.5 M of sodium chloride. The eluate is neutralized with 0.1N NaOH to obtain pH 7.5 and then sterilized (0.22 μm Gelman™ filter) and frozen at -20°C.

Claimed is:

1. Human lactoferrin expressed by recombinant DNA.

2. Lactoferrin having less than 25% metal loading.

3. The lactoferrin of claim 2 that is human lactoferrin.

4. The lactoferrin of claim 2 having less than 10% metal loading.

5. Lactoferrin having at least 35% metal loading.

6. The lactoferrin of claim 5 having at least 50% metal loading.

7. The lactoferrin of claim 5 that is human lactoferrin.

8. A genetically altered organism capable of producing human lactoferrin.

9. The organism of claim 8 that is eukaryotic.

10. The organism of claim 8 that is Pichia pastoris.

11. A process for producing human lactoferrin comprising the steps of:

(a) isolating DNA encoding human lactoferrin from a cDNA library;

(b) inserting the isolated DNA into the DNA of a host organism;

(c) culturing the host organism to express human lactoferrin; and

(d) recovering the expressed human lactoferrin from culture media.

12. The process of claim 11 wherein the lactoferrin is recovered by the steps of (i) filtering the culture media to retain a first material having a molecular weight of at least 10,000 daltons, (ii) chromatographing the retained material on an adsorbent having chromophore C.I. 61211 as an affinity ligand to obtain adsorbed and non-adsorbed fractions, (ii) chromatographing the adsorbed fraction on a medium wherein the medium is controlled pore glass beads or silicic acid to obtain adsorbed and non-adsorbed fractions, and (iii)

filtering the adsorbed fraction from the medium to retain a second material having a molecular weight of at least 10,000 daltons.

13. The process of claim 12 further comprising the steps of (iv) chromatographing the second retained material on T-Gel at least once to obtain adsorbed and non-adsorbed fractions, and (v) filtering the non-adsorbed fraction from the T-Gel to retain a third material having a molecular weight of at least 10,000.

14. The process of claim 12 further comprising the steps of (iv) chromatographing the second retained material on a third adsorbent having phenyl glycidyl ether as an affinity ligand to obtain adsorbed and non-adsorbed fractions, and (v) filtering the adsorbed fraction from the third adsorbent to retain a third material having a molecular weight of at least 10,000 daltons.

15. The process of claim 12 further comprising the steps of (iv) chromatographing the second retained material on a fourth adsorbent having an immobilized metal ion as an affinity ligand to obtain adsorbed and non-adsorbed fractions, and (v) filtering the adsorbed fraction to retain a fourth material having a molecular weight of at least 10,000 daltons.

16. A process for inhibiting microbial growth on a mammal comprising topically applying a therapeutically effective amount of lactoferrin having less than 25% metal loading.

17. The process of claim 16 wherein the lactoferrin is human lactoferrin expressed by recombinant DNA.

18. A process for inhibiting trace-element deficiency in a mammal comprising orally administering a nutritionally effective amount of lactoferrin having at least 35% trace-element loading.

19. The process of claim 18 wherein the lactoferrin is human lactoferrin expressed by recombinant DNA.

20. The process of claim 18 wherein the trace element is at least one of iron, copper, or zinc.

21. A method for retarding food-spoilage comprising applying to food an effective amount of lactoferrin having less than 25% metal loading.

22. The method of claim 21 wherein the lactoferrin is human lactoferrin expressed by recombinant DNA.

23. A nutritional supplement comprising an effective amount of lactoferrin having at least 35% trace-element loading and a nutritionally acceptable carrier or diluent.

24. The supplement of claim 23 wherein the lactoferrin is human lactoferrin expressed by recombinant DNA.

25. The supplement of claim 23 wherein the trace element is at least one of iron, copper, or zinc.

26. A disinfectant comprising an effective amount of lactoferrin having less than 25% metal loading and an acceptable carrier or diluent.

27. The disinfectant of claim 26 wherein the lactoferrin is human lactoferrin expressed by recombinant DNA.

28. A food-spoilage retardant comprising an effective amount of lactoferrin having less than 25% metal loading and an acceptable carrier or diluent.

29. The additive of claim 28 wherein the lactoferrin is human lactoferrin expressed by recombinant DNA.

30. In a chromatography process comprising the steps of (a) contacting a substance with a first adsorbent to obtain adsorbed and non-adsorbed fractions, (b) eluting the adsorbed fraction with an eluant, and (c) contacting the adsorbed fraction with a second adsorbent, the improvement comprising equilibrating the second adsorbent with the eluant followed

by contacting the eluate containing the adsorbed fraction with the second adsorbent.

31. The process of claim 30 wherein the first adsorbent is contained in a first column connected to a second column containing the second adsorbent by a means through which the eluate passes from the first column to the second column.

32. The process of claim 31 wherein the means sequentially (i) discharges material not adsorbed on the first adsorbent from the first column and (ii) passes the eluate from the first column to the second column.

33. The process of claim 32 further comprising the sequential steps of (d) discharging non-adsorbed material from the second-adsorbent, (e) eluting adsorbed material from the second adsorbent, and (f) filtering the eluate, wherein the non-adsorbed material and the eluate pass through a means capable of directing the effluent from the second column to the filtering means or the discharge locus.

34. The process of claim 31 further comprising the steps of obtaining the substance by passing a mixture through a filter that retains the substance, and prior to contacting the substance with the first adsorbent, washing the substance on the filter with a buffer and equilibrating the first adsorbent with the buffer.

35. A chromatography apparatus comprising (a) a first column containing a first adsorbent, (b) a second column containing a second adsorbent, and (c) a means capable of (i) carrying effluent from the first column to the second column or (ii) discharging the effluent.

36. The apparatus of claim 35 further comprising (d) means capable of directing effluent from the second column to at least two alternative loci.

37. In a chromatography process comprising the steps of (a) equilibrating an adsorbent with a buffer and (b) applying a protein-containing material to the adsorbent to obtain adsorbed and non-adsorbed fractions, the improvement comprising passing the material through a filter capable of excluding material having a molecular weight greater than 10,000 daltons and washing the material on the filter with the buffer before applying the material to the adsorbent.

38. A process for producing human lactoferrin comprising the steps of:

(a) culturing a host cell genetically altered to carry DNA encoding human lactoferrin to express human lactoferrin and

(b) recovering the expressed lactoferrin from culture media.

39. Genetically modified DNA capable of expressing human lactoferrin in a host organism.

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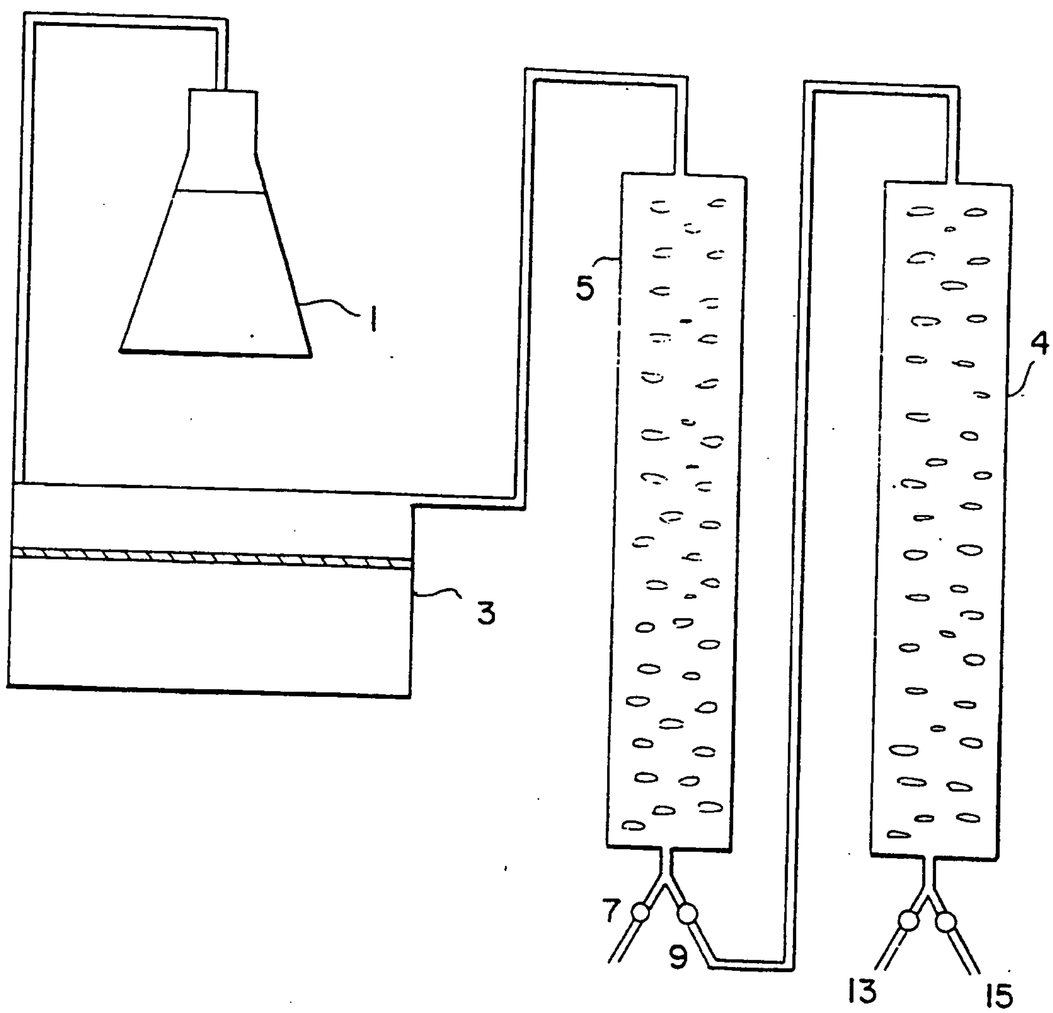
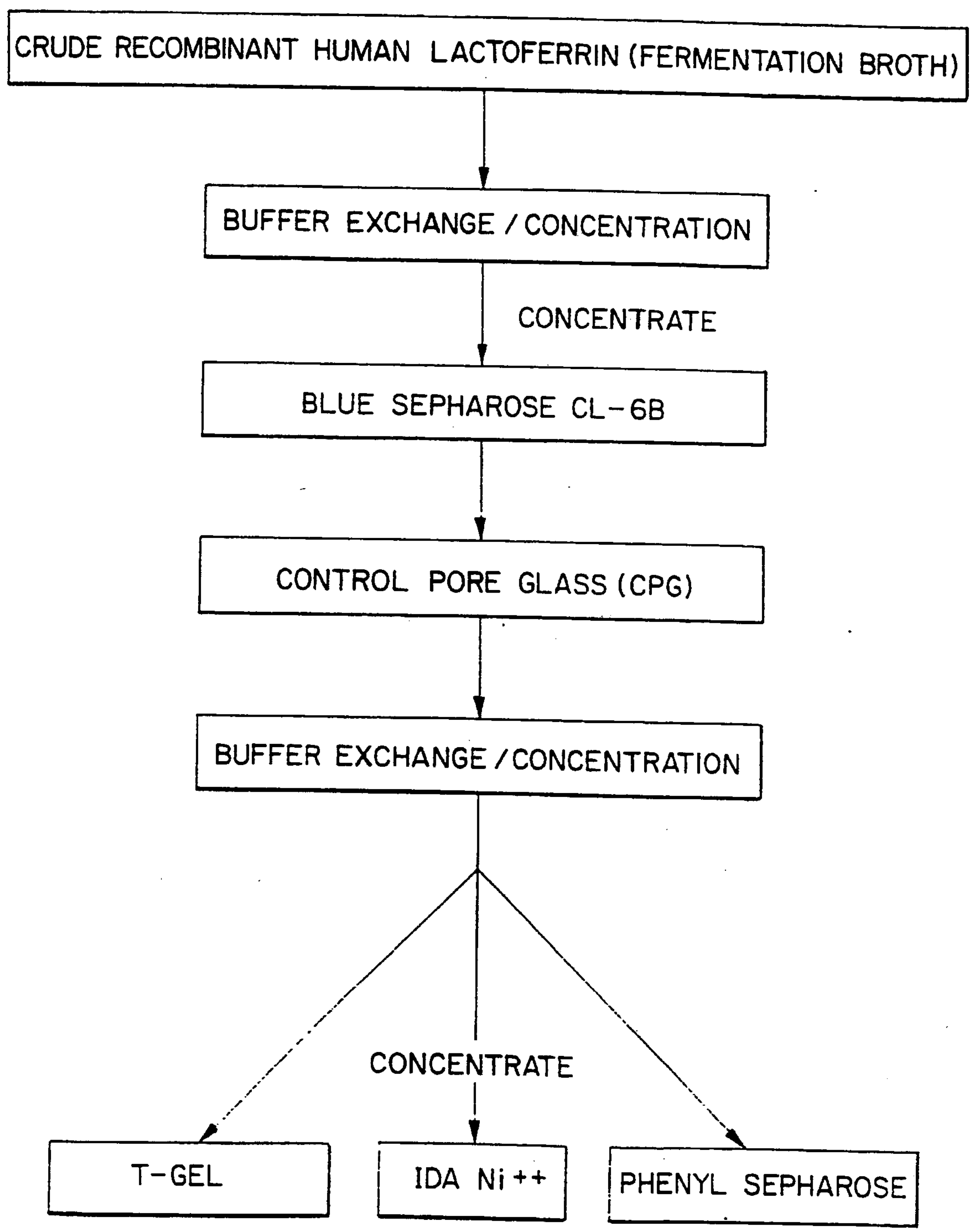
FIG. 1



SUBSTITUTE SHEET

FIG. 2


CRUDE RECOMBINANT HUMAN LACTOFERRIN (FERMENTATION BROTH)
BUFFER EXCHANGE / CONCENTRATION
CONCENTRATE
BLUE SEPHAROSE CL-6B
CONTROL PORE GLASS (CPG)
BUFFER EXCHANGE / CONCENTRATION
CONCENTRATE
T-GEL
IDA Ni ++
PHENYL SEPHAROSE

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(54) **Use of lactoferrin for therapy of acute or recurrent infectious diseases by streptococcus pyogenes or other intracellular gram positive pathogen bacteria**

(57) The present invention relates to the therapeutic utilization of the protein lactoferrin for the therapy of many acute or recurrent bacterial infectious diseases of men and animals. In details, the present invention demonstrates an anti-invasive property of lactoferrin in apo and iron-saturated form towards intracellular Gram positive pathogen microorganisms, like *Streptococcus pyogenes* and *Staphylococcus aureus* not being related to the well known antibacterial activity of lactoferrin and to the other known properties of the protein. The same anti-invasive activity of lactoferrin is also possessed, even if to a lower extent, by polypeptide fragments of the protein and by other transferrin proteins eventually chemically modified.

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Description

TECHNICAL FIELD

The present invention relates to the therapeutic utilization of lactoferrin for preventing and curing many bacterial infectious diseases of men and animals, caused by intracellular Gram positive pathogen microorganisms.

BACKGROUND ART

A first step of bacterial infection can be represented by the adhesion of bacteria to the host cell. Some pathogen bacteria are able to adhere to the cell through specific surface molecules while others are to invade the cell, to survive in the endosome or, escaping from the vacuole, to replicate within the cytoplasm. The molecules that allow bacteria to internalize into the cells are considered virulence factors because bacteria in intracellular phase, evade the host immune system and drugs. Intracellular bacteria can multiply in the cell, causing an acute infection, or can survive into the cell recuring in some circumstances. This last event can explain the recurrence of bacterial infections of ephitelium and mucosas. An example of this phenomenum is well represented by the recurrent infections of oropharynx caused by *Streptococcus pyogenes*. Group B-hemolytic Streptococci are responsible of several diseases such as erysipelas, puerperal fever, sepsis, streptococcical angina, impetigo, genital-urinary infections, rheumatic fever and nephritis. In particular, streptococcical angina, caused by Group A-Streptococci, is a recurrent infection very usual in children. These bacteria exert their pathogenicity by colonizing the pharynx epithelium adhering to host cells with specific molecules and producing in the human host rhinopharyngitis, tonsillitis, mucous edema, swelling and ache of cervical lymphonodes. Even after an antibiotic treatment (usually penicillin or erytromycin), it has been widely demonstrated the appearance of a lot of recurrent infectious diseases. In fact, following an appropriate antibiotic therapy owing to the patient full clinical recovery (this occurrence can be supported by a pharyngeal isolate negative for *Streptococcus pyogenes*) is possible to have an reinfection in 2-4 weeks.

The property of *S. pyogenes* to cause recurrent infection diseases can be now attribuite to the invasive capability, recently demonstrated, by which this bacterium can adhere and enter epithelial cells avoiding any effect either from the immune system of the host or from an antibiotic treatment. It has been also demonstrated the capability of *Staphylococcus aureus* to invade host epithelial cells. In fact, this pathogen bacterium colonizes human skin and causes suppurative infectious diseases. *Staphylococcus aureus* is also responsible of deep infections such as abscesses, osteomyelitis, clinical treatment pyelonephritis, penumonia, empyema, purulent arthritis, septicemia and endocarditis.

It is therefore possible to understand the importance of therapeutic treatment in order to preventing and inhibit the adhesion and the invasion of epithelial cells by *Streptococcus pyogenes* and other Gram positive invasive microorganisms. This therapy, hindering the bacterial internalization, will help the antibiotic treatment and, at the same time, will prevent the recurrence of infections. Up to now, there are not drugs on market that can be utilized for this purpose in humans and animals.

DISCLOSURE OF THE INVENTION

The therapeutic model, described in the present invention, is based on the utilization of the anti-invasive activity of lactoferrin (or its analogous molecules or its fragments) towards bacteria. This anti-invasive property of lactoferrin represents a new approach for the therapy of infectious diseases caused by intracellular Gram positive pathogen bacteria. Lactoferrin topic treatment is characterized by very low or no toxic effects and, therefore, can be utilized in bacterial infections concerning skin and mucosas like oropharynx, nasal, intestinal, bronchial, vaginal or other mucosas. The therapeutic utilization of this anti-invasive property possessed by lactoferrin is not related to the antibacterial activity of the molecule and, in a more general way, the antiinvasive property, is possessed by proteins analogous to lactoferrin, generically called transferrins, and is also possesed by fragments of these proteins even to a lesser extent. Since at the present time there are no *in vivo* models for testing the anti-invasive properties of substances towards *S. pyogenes*, the examples below described have been performed to *in vitro* models that are generally accepted for assaying anti-invasive molecules. This anti-invasive activity possessed by lactoferrin (or its analogous molecules or its fragments) towards invasive bacteria is well demonstrated *in vitro* by the lawer number or absence of *S. pyogenes* inside cultured epithelial human HeLa cells, when lactoferrin at non cytoxicic and non bactericidal concentrations (e.g. up to 2mg/ml) is present at the same time with the cells and the microorganisms.

Furthermore, it has been demonstrated that lactoferrin possesses an anti-invasive activity towards other intracellular Gram-positive pathogen bacteria like *Staphylococcus aureus*, indicating a therapeutic use of lactoferrin in order to prevent and cure other acute or recurrent infections dependent on the internalization of a Gram positive pathogen microorganism in the host cells.

WAYS OF CARRYING OUT THE INVENTION

Lactoferrin from bovine milk is the particulary preferred protein according to the invention. The examples, below reported, show that bovine lactoferrin can be considered optimum, with respect to treatments currently in use, for the prevention and cure of recurrent infectious diseases from intracellular Gram positive pathogen microorganisms. Furthermore, the extremely low toxicity of said protein contained in the formula according to the present invention is well-known, since it is a "natural" substance extracted from bovine milk and is very similar, in its amino acid composition and structural configuration, to the homologous protein that is normally present in humans. This is an additional advantage of bovine lactoferrin with respect to the chemicals currently used in infection treatments. The protein that is used according to the present invention can be extract or can be obtained by recombinant-DNA technology or also by chemical synthesis.

The term lactoferrin designates a glycoprotein present in colostrum and milk, in many biological secretions and in the leucocyte granules of mammalians. Lactoferrin possesses an isoelectric point of 7.8, a molecular weight of about 83,000 Da, two sugar chains and a variable saturation with iron. Each molecule of lactoferrin binds 2 atoms of Fe^{3+} in the presence of sodium bicarbonate. The main function of this protein is to reduce the amount of free iron in biological liquids, inhibiting the bacterial growth (the bacteriostatic property of lactoferrin is already well known) and decreasing the risk of forming free radicals due to the presence of not chelated iron. Lactoferrin belongs to a family of glycoprotein generally called "Transferrin", characterized in so that they possess two binding sites for Fe^{3+} per molecule and a high degree of homology among them. Bovine lactoferrin can be industrially produced by purification from bovine milk.

The therapeutic utilization of the anti-invasive activity of lactoferrin towards intracellular Gram-positive pathogen bacteria is the main claim of this disclosure while it is not relevant, as regard with the anti-invasive activity, the source of the lactoferrin (i.e. from extractive chemistry or from recombinant DNA technique).

The anti-invasive activity described in the present disclosure, is possessed by all the marketable proteins known as lactoferrin such as human lactoferrin or mouse lactoferrin and by other proteins belonging to the "Transferrin family" (even if they show a lesser anti-invasive activity) produced by extraction or utilizing recombinant DNA technique, and, therefore, the anti-invasive activity of lactoferrin has to be extended, as described in the present disclosure, to all the lactoferrins, from various sources, to transferrins and related proteins, from various natural or industrial sources.

The anti-invasive activity of lactoferrin has been showed at not toxic concentrations of lactoferrin towards human cultured cells and at non antibactericial concentrations towards the tested microoganisms; all the preparation of lactoferrin with different iron saturation from apo-form to totally Fe^{3+} saturated form, showed the same antiinvasive property. The antiinvasive activity of lactoferrin towards microorganisms, whose therapeutic utilization is described in the present disclosure, is also possessed by its fragments, obtained by chemical and enzimatic treatments or obtained through chemical synthesis or other technologies like recombinant DNA technique.

The lactoferrin activity according to the present invention can be obtained using lactoferrin stored in liquid form, as solution at concentration of 0.1 to 10%, e.g. 1 to 5%, weight/volume (g/ml) of the protein in solvents accettable for pharmaceutial use, in particular water or hydro-alcoholic solvents such as water-ethanol mixtures, or in solid form (lyophilized, dried, frozen) and in the other commonly known forms of storage: for example immobilized or adsorbed on an inert support commonly used in the pharmaceutical field.

Lactoferrin, with respect to the activity according to the invention, can thus be used in the liquid form, as a rinse or gargle, or in solid form, such as granular form to be dissolved in water for preparing a gargle just before use, with the concentrations specified above or also in powder for use on skin. Alternatively, it is possible to incorporate the protein mixture of the invention in a formulation to be chewed, such as chewing gum, tablets, pastilles, lozenges, etc. in a concentration of 1 to 60% by weight of the total formulation.

In its form ready-for-use, the lactoferrin formulation with respect to the activity according to the invention, can also comprise further conventional antibiotic and antibacterial compounds as well as carries, fillers, flavouring agents, preservatives, surfactants, colorants and other adjuvants selected from those conventionally used for the various liquid or solid form preparations for oral topical use and for skin topical use.

Thus, lactoferrin formulation, with respect to the activity according to the invention, can also comprise antibiotic compounds like: penicillins, cephalosporins, chloromphenicol, macrolids, aminoglicosids, sulphamidics etc. and further anti-bacterial compounds, such as quaternary ammonium compounds with one long chain alkyl on the nitrogen atom, alkali metal pyrophosphates and orthophosphates, halogenated bisphenols and halogenated diphenyl ethers, sodium benzoate, sodium salicylate, etc.

Lactoferrin formulation, with respect to the activity according to the invention, can further comprise humectants, e.g. glycerin, sorbitol, xylitol, propylene glycol, atc., flavours, e.g. oil of spearmint; peppermint or cinnamon, menthol, methyl salicylate, atc., sweetening agents, e.g. aspartame, saccharin, dextrose, cyclamate, wintergreen, etc., thickening agents, e.g. xantan gum, carrageenin, carboxy methyl cellulose, etc..

The formulation to be chewed by the user will comprise the respective conventional base material and conventional adjuvant such as flavouring, sweetening and coloring agents, humectants, etc., as those mentioned above; and thickening and gelling agents such as thickening silica, natural or synthetic gums, e.g. tragacanth gum, guar gum, hydrox-

yethyl- and, carboxymethyl cellulose, polyvinyl pyrrolidone, starch, etc..

Lactoferrin formulation, with respected to the activity according to the invention, in either liquid or solid form, should be used for purposes of prevention of recurrent infectious diseases by *S.pyogenes* or by *S.aureus* at last once, preferably twice a day; for purposes of treatment the frequency of use can be increased to 3 to 4 times a day.

The following examples give a strong evidence of the therapeutical utilization of antiinvasive activity of lactoferrin (or its analogous molecules or its fragments) towards intracellular Gram positive pathogen microorganisms. This utilization, described in the present disclosure, can be considered optimal as regards with the common therapeutical treatments, because of its utilization alone or as adjuvant in antibacterial therapies concerning acute or recurrent infections by intracellular Gram positive pathogen bacteria.

EXAMPLES

The evaluation of the invasive capability of *S. pyogenes* must occur through a specific experimental procedure that we described below:

Bacterial strains and cultures

Streptococcus pyogenes strain 95/1 was cultured in Todd-Hewitt broth (Difco lab.). Blood agar was used for counting coloning forming units (C.F.U.).

Staphylococcus aureus strain 94/3 was cultured in Brain Hearth Infusion (BHI). Mannitol Salt Agar was used for C.F.U. counts.

HeLa S3 human epitelial cells (derived from epithelioid carcinoma of human cervix) were grown as monolayers at 37°C in Eagle's MEM containing 1.2 g/l of $NaCo_3$, 2 mM glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 10% heat inactivated foetal calf serum in 5% CO_2 on 24-well tissue culture clusters (costar) by loading 1 ml of a solution of $1.5x10^5$ cells/ml.

Invasion assay and test for anti-invasive activity of lactoferrin

Semiconfluent monolayers of HeLa cells were infected with a bacterial suspension (at a multiplicity of infection of 100 exponentially grown bacteria per cell) deriving from a bacterial subculture after an incubation at 37°C for 120 min in the presence and absence of lactoferrin added at no cytotoxic nor antibacterial concentrations. After the bacterial infection (2 h at 37°C), the monolayer was five-fold carefully washed with fosfate buffered saline (PBS) and then fresh medium (MEM, Seromed) containing 200 ug/ml of gentamicin was added. This antibiotic is well known for its impossibility to penetrate into the eukaryotic cells and to exert its bactericidal effect only towards the extracellular bacteria (free or adhered to the cells). After 2 h incubation period at 37°C in gentamicin, the infected cells were whashed three times with PBS and lysed by the addition of cold 0.1% Triton-X 100.

The cellular lysate was diluited with PBS and plated on selective agar medium in order to determine viable intracellular bacteria.

EXAMPLE 1

Anti-invasive activity of lactoferrin towards *S. pyogenes* 95/1 at non cytotoxic and non bacterial concentrations.

First of all, we evaluated the minimal toxic concentration towards the cells. Different lactoferrin concentrations were kept in contact with cellular monolayers. After 24 h incubation period, the monolayers were examined by optical microscopy after vital staining.

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Table 1

Toxicity of different concentrations of lactoferrin towards HeLa cells.	
Lactoferrin (mg/ml)	HeLa cells
0	-
0,25	-
0.50	-
1.00	-
2.00	-
4.00	-
8.00	-
50.00	+
The cytotoxic effect was evaluated by examining the cell morphology and vitality (-) no cytotoxic effect (+) presence of cytotoxicity	

The lactoferrin was also tested in order to quantify the minimal concentration not inhibiting the bacterial growth.

Table 2

Antibacterial activity of lactoferrin towards *S. pyogenes* 95/1.	
Lactoferrin (mg/ml)	*S.pyogenes* (CFU/ml)
0	2×10^8
0.5	3×10^8
1.0	2×10^8
2.0	2×10^8
5.0	5×10^7
10.0	1×10^7

The antibacterial activity of lactoferrin was carried out in Todd Hewith broth incubated at 37°C for 18 h. After this period, the aliquots of the mixture were plated on Blood agar and the viable bacteria were quantified by CFU counts. The inoculum consisted of about 5×10^5 cell/ml.

The anti-invasive effect of lactoferrin at non cytotoxic and non bactericidal concentration (1 mg/ml) was tested utilizing *S. pyogenes* 95/1 added to HeLa cell monolayers.

Table 3

Effect of lactoferrin on the invasion ability of *S. pyogenes* 95/1	
Lactoferrin (mg/ml)	Internalized bacteria (CFU/ml)
0	5×10^5
1.0	5×10^3
2.0	1×10^3

The anti-invasive effect of lactoferrin is demonstrated by a 100/500-fold decrease of the number of internalized bacteria.

The anti-invasive effect of lactoferrin has been also compared with that obtained utilizing ovotransferrin, a protein analogous to lactoferrin, at non cytotoxic and non bacterialidal concentrations. The data are showed in Table 4.

Table 4

Anti-invasive activity of ovotransferrin in comparison with lactoferrin towards *S. pyogenes* 95/1		
Lactoferrin (mg/ml)	Ovotransferrin (mg/ml)	Internalized bacteria CFU/ml)
0	0	5×10^5
1.0	0	5×10^3
0	1.0	1×10^5

The Table 4 shows that the anti-invasive effect of ovotransferrin is much lesser than that observed with lactoferrin. All the molecules, generally called transferrins, possess, even if in a different degree, an anti-invasive activity towards *S. pyogenes* 95/1.

It must be underlined that the above mentioned anti-invasive activity of lactoferrin and other transferrins is not affected by the degree of iron saturation of these proteins.

EXAMPLE 2

Anti-invasive activity of lactoferrin towards *S. pyogenes* 95/1 in presence of subinhibiting concentrations of antibiotics.

It is well known as *S. pyogenes* 95/1 is sensible to the antibacterial action of erythromycin and ampicillin. Subinhibiting amounts of erythromycin or ampicillin were utilized in order to verify if the bacteria pretreated with this antibiotic are inhibited in their invasion efficiency.

For this reason, *S. pyogenes* 95/1 was cultured in presence of subinhiting concentrations of antibiotics and the invasivity test was performed with this bacterial inoculum. The data are reported in Table 5.

Table 5

Effect of subinhibiting concentrations of antibiotics towards the invasivity of *S. pyogenes* 95/1 in absence of lactoferrin.	
Antibiotics (ug/ml)	**Internalized bacteria (CFU/ml)**
0	5×10^5
Erythromicin 20	2×10^5
Ampicillin 0.1	2×10^5

The reported data demonstrate that bacteria pretreated with antibiotics do not lose their invasion capability.
It was tested, therefore, the anti-invasive activity of lactoferrin towards *S. pyogenes* 95/1 pretreated with subinhibiting amounts of erythromycin or ampicillin. The effect of lactoferrin was tested towards *S. pyogenes* 95/1 grown for 24 h in presence of erythromycin (20 ug/ml) or in presence of ampicillin (0.1 ug/ml). The results are showed in Table 6.

Table 6

Anti-invasive activity of lactoferrin towards *S. pyogenes* 95/1 pretreated with antibiotics.		
Lactoferrin (mg/ml)	**Antibiotic (ug/ml)**	**Intracellular bacteria (CFU/ml)**
0	0	5×10^5
1	0	6×10^3
2	0	4×10^3
1	Erythromicin 20	3 x 10
2	Erythromicin 20	0
1	Ampicillin 0.1	0

The same test was performed with other antibiotics and fairly similar results were obtained. It is well evident that the anti-invasive activity of lactoferrin increases when bacteria are pretreated with antibiotics, demonstrating the co-operative effect of a therapeutic treatment of lactoferrin and antibiotics against infectious diseases by intracellular pathogen bacteria.

EXAMPLE 3

Anti-invasive activity towards *S. pyogenes* 95/1 possessed by lactoferrin and ovotransferrin chemically or enzimatically modified.

The performed modifications are described below:

- the fragment 1 was obtained by treating the lactoferrin with a mixture of proteolytic enzimes without any further purification. The electrophoresis analysis showed the presence of different fragments with a molecular weight lesser than the untreatred lactoferrin and the absence of intact lactoferrin;
- the fragment 2 was obtained by treating the lactoferrin with a mixture of enzymes endoglycosidases and esoglycosidases without any further purification. The analysis of glucides showed a decrease of about 70% of the glycosilation degree of lactoferrin.
- the glycosilate ovotransferrin was obtained by incubating the protein at alkaline pH with high glucide concentrations, according with the common methods to increase the glycosilation of proteins.

Table 7

Anti-invasive activity towards *S.pyogenes* 95/1 possessed by modified transferrins utilized at the concentration of 1 mg/ml.	
Protein	**Intracellular bacteria (CFU/ml)**
None (control)	5 x 10^5
Lactoferrin	5 x 10^3
Ovotransferrin	1 x 10^5
Fragment 1	5 x 10^4
Fragment 2	8 x 10^4
Glycosilate ovotransferrin	1 x 10^4

From the above Table is evident that all the modified preparation show an anti- invasive activity.

EXAMPLE 4

Protective activity of lactoferrin towards the cell penetration of other intracellular pathogen Gram positive bacteria.

The anti-invasive activity of lactoferrin towards other intracellular pathogen Gram positive bacteria has been showed by means of the described protocol.

Table 8

Anti-invasive activity of lactoferrin, its analogous and fragments (at the concentration of 1 mg/ml) also in presence of subinhibiting amounts of erythromycin (0.02 mg/ml) towards intracellular pathogen Gram positive bacteria.		
Proteins	**Intracellular bacteria (CFU/ml**	
	S. pyogenes	*S. aureus*
None (Control)	5 x 10^5	7 x 10^5
Erythromycin	2 x 10^5	3 x 10^5
Apo-lactoferrin	5 x 10^3	6 x 10^3
Iron saturated lactoferrin	5 x 10^3	6 x 10^3
Fragment 1	5 x 10^4	4 x 10^5
Fragment 2	8 x 10^4	3 x 10^5
Glycosilate Ovotransferrin	1 x 10^4	1 x 10^4
Lactoferrin + Erythromycin	0	0
Lactoferrin + Ampicillin	0	0
Fragment 1+ Erythromycin	2 x 10^2	2 x 10^3
Fragment 1 + Ampicillin	1 x 10^2	3 x 10^3
Fragment 2 + Erythromycin	3 x 10^2	3 x 10^3
Fragment 2 + Ampicillin	1 x 10^2	2 x 10^3
Glycosilate Ovotransferrin + Erythromicin	1 x 10	8 x 10
Glycosilate Ovotransferrin + Ampicillin	2 x 10	6 x 10

The experiments reported in Table 8 demonstrate that the lactoferrin and its analogous, such as ovotransferrin and glycosilate ovotransferrin and its fragments such as the fragment 1 above described, are active towards all the examined intracellular Gram positive pathogen bacteria and that this activity is increased in presence of subinhibiting amounts of antibiotic molecules.

EXAMPLE 5 -Gargle

Composition per 100 ml

Active ingredients :

Lactoferrin, from bovine milk, SIGMA Chemical Co, cat L 4765, 1.0 g.

Carriers, preservatives and flavouring agents:

Sodium chloride 1 g
Sodium bicarbonate 100 mg
Methyl-p-hydroxybenzoate 100 mg
Peppermint oil 50 mg
Purified water to 100 ml

Use:

For prevention and curing Streptococcical angina two gargle a day, one in the morning, one in the evening.

EXAMPLE 6 - Envelope packs

Composition for 1 envelope

Active ingredients:

Lactoferrin, from bovine , SIGMA Chemical Co., cat L4765, 1.0 g.
Erytromicin 0.2 g

Carriers, preservatives and flavouring agents:

Sodium choloride 20 mg
Sodium bicarbonate 10 mg
Methyl-p-hydroxybenzoate 20 mg
Peppermint oil 5 mg

Use:

For preventing and curing Streptococcical angina, the content of an envelope is dissolved in 20 ml water for two rinse, one in the morning and one in the evening.

EXAMPLE 7 - Chewing gum

Composition of one piece of gum :

Active ingredients:

Lactoferrin, from bovine milk, SIGMA Chemical Co. cat L4765, 250 mg.
Benzalkonium chloride 10 mg

Carries, preservatives and flavouring agents:

Gum base (Paloya TX) 400 mg
Glucose 100 mg

Glycerol 10 mg
Sodium bicarbonate 10 mg
Methyl-p-hydroxybenzoate 20 mg
Peppermint oil 5 mg

Use:

For preventing and curing Streptococcical angina, one piece of chewing gum in the morning, one in the evening.

EXAMPLE 8 - Powder

Composition for 100 g

Active ingredients:

Lactoferrin from bovine milk, SIGMA Chemical Co., cat L 4765, 10 g
ampicillin 1g

Carriers, preservatives

corn starch 85 g
zin oxide 3 g
thickening silica powder 1 g

Use:

For curing staphilococcical infections of skin apply twice a day.

Claims

1. Therapeutic utilization of lactoferrin for preventing or treating infectious diseases by intracellular Gram positive pathogen bacteria. Lactoferrin for therapeutic utilizzation in preventing and treating infectious diseases by intracellular Gram positive pathogen bacteria characterized in that said protein inhibits invasion of said microorganisms into host cells.

2. Therapeutic utilization of the anti-invasive activity of lactoferrin, according to claim 1, utilized for preventing or treating infectious diseases by *Streptococcus pyogenes* concerning oropharynx of infants and adults and for preventing and treating related recurrent infectious diseases in humans.

3. Therapeutic utilization of the anti-invasive activity of lactoferrin according to claim 1, towards other intracellular Gram positive pathogen bacteria such as *Staphylococcus aureus*, for preventing or treating acute and recurrent bacterial infections concerning skin and different type of mucosas such as oropharynx, nasal, intestinal, bronchial, vaginal or other mucosas.

4. Therapeutic utilization of the anti-invasive activity of lactoferrin towards *Streptococcus pyogenes* and other intracellular Gram positive pathogen bacteria, according to claims 1,2 and 3, possessed by lactoferrin with a variable saturation in Fe^{3+}.

5. Therapeutic utilization of the anti-invasive activity of lactoferrin towards *Streptococcus pyogenes* and other intracellular pathogen Gram positive bacteria, according to claims 1,2 and 3, possessed by lactoferrin or by proteins or peptides characterized by high sequence omology with lactoferrin like native or chemically modified transferrins, extracted by natural sources: animals or vegetables, or produced by chemical synthesis or by other technology such as recombinant DNA tecniques.

6. Therapeutic utilizzation of the antiinvasive activity of lactoferrin, according to claims 1,2,3,4 and 5, when said protein is in liquid preparation, solid preparation and preparation immobilized or adsorbed on an inert support.

7. Therapeutic utilizzation of the antiinvasive activity of lactoferrin, according to claims 1,2,3,4 and 5, when said protein is in liquid preparation, solid preparation and preparation immobilized or adsorbed on an inert support, in a gar-

gle formulation, envelope packs, chewing gum or powder in a 0,1 to 10% by weight.

8. Therapeutic utilizzation of the antiinvasive activity of lactoferrin, according to claims 1,2,3,4 and 5, when the protein is in liquid prearation, solid preparation and preparation immobilized or adsorbed on inert support, associated to one or more antibiotics or antibacterial agents.

9. Therapeutic utilizzation of the antiinvasive activity of lactoferrin, according to claims 1,2,3,4 and 5, the protein being in liquid prearation, solid preparation and in preparation immobilized or adsorbed on inert support, associated to one or more adjuvants selected from humectants, sweeteners, flavouring agents, thckening agents acceptable for topical use on skin or mucosas.

10. Methods of using lactoferrin, according to claims 1,2 and 3 for preventing or treating bacterial infections comprising topically administering said protein to the mucosas or skin at least once a day.

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(54) Preparation of lactoferrin (or analogous proteins) and desferrioxamine methanesulfonate (or other metal ion chelators) for the therapy of viral infectious diseases

(57) The present invention relates to the therapeutic utilizzation of the preparation of lactoferrin and desferrioxamine metalsulphonate for the therapy of many acute or recurrent viral infectious diseases in humans and animals. In details, the present invention demonstrates the antiviral activity, based on the inhibition either of the absorption either of the replication of several virus, possessed by a preparation of lactoferrin (or its anologous proteins like transferrins) in apo or iron or other metal ions saturated forms, together with desferrioxamine metansulfonate (or other metal ion chelators like 8-idroxiquinoline 1,10-phenanthroline, phosphonoacetic acid). This antiviral activity is well evident towards DNA virus; like *Herpesviruses*, and towards RNA virus, like *Rhinovirus*, and can be generally extended and utilized for the therapy of many acute or recurrent viral infections concerning skin, mucosas or other tissues.

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toms but is not able to prevent the recurrence of the infections.

Rhinovirus are casual agents of the common cold. For this virus it is not possible the formulation of an effective vaccine, because of the 115 antigenic varieties found. The relative harmlessness of the above viral infections does not suggest the therapeutic utilization of antiviral drugs that always possess toxicity towards the host.

DISCLOSURE OF THE INVENTION

The present disclosure concerns the preparation of a mixture of at least one transferrin like lactoferrin (and, in a more general way, of proteins analogous to lactoferrin) and of at least one metal ion chelators such as desferrioxamine methanesulfonate or 8-hydroxyquinoline, 1,10-phenanthroline, phosphonoacetic acid, whose antiviral activity, that is here demonstrated to be exerted through the inhibition of adsorption and replication of several viruses, can be therapeutically utilized.

The therapeutic model, described in the present invention is based on the synergistic antiviral action of lactoferrin (or analogous proteins) and desferrioxamine metansulfonate (or other metal ion chelators). The antiviral activity of this association, not yet described, is much higher than that showed separately by the two components because their antiviral activity is exerted on two different phases of the viral cycle (adsorption and replication). It, therefore, makes obvious the therapeutic benefit of such an association towards viral infectious diseases in comparison with the common antiviral therapies that are focused on the inhibition of viral replication and show toxic effects towards the host organism.

Lactoferrin, which possesses very low or no toxic effect, and the chelator desferrioxamine methanesulfonate, already utilized in the human therapy, can be used at topic and systemic level against viral infections concerning skin and mucous membranes like nasal, oropharynx mucosa, intestinal, bronchial, vaginal and other mucosas.

As an example, we showed the antiviral activity towards DNA and RNA viruses, possessed by the preparation of lactoferrin (or its analogous proteins) and desferrioxamine metansulfonate (or other metal ion chelators). The antiviral activity is made clear by the protection of cell lysis due to viral replication of HSV1 (choosed as DNA virus) and *Rhinovirus* (choosed as RNA virus). The antiviral activity of the preparation of lactoferrin (or its analogous proteins) and desferrioxamine metansulfonate (or other metal ion chelators), is made evident by the absence or reduced presence of lysis plaques when the virus is added to cells cultured in the presence of this association. Since the antiviral activity of the preparation of between lactoferrin (or its analogous proteins) and desferrioxamine metansulfonate (or other metal ion chelators) is exerted towards either DNA viruses (like HSV1) either RNA viruses (like *Rhinovirus*,), is pointed out the possibility of an utilization of this association also for preventing and treating other acute or recurrent viral infections.

WAYS OF CARRYING OUT THE INVENTION

A particularly preferred preparation according to the invention is a mixture of the two compounds mentioned above, and thus a mixture of lactoferrin at a concentration of 15 to 99% methansulfonate and desferoxamine at a concentration af 1 to 95% these concentration being given by weith respect to the mixture. By way of example, a preparation according to the invention can comprise 98% lactoferrin and 2% desferoxamine methansulfonate.

Lactoferrin from bovine milk is the particularly preferred protein according to the invention. It appears clear, from the example reported below, that bovine lactoferrin can be considered optimum, with respect to treatments currently in use for the prevention and cure of viral infectious diseases although can be substituted by recombinant human lactoferrin in the case of systemic treatments. Furthermore, the extremely low toxicity of said protein contained in the formula according to the present invention is well-known, since is a "natural" substance extracted from bovine milk and is very similar, in its amino acid composition and structural configuration, to the homologous protein that is normally present in humans. This is an additional advantage of bovine lactoferrin with respect to the chemicals currently used in infections treatments. The protein that is used according to the present invention can be extract or can be obtained by recombinant-DNA technology or also by chemical synthesis.

The term lactoferrin designates a glycoprotein present in colostrum and milk and in many biological secretions and in the leucocyte granules of mammalians. Lactoferrin possesses an isoelectric point of 7.8, a molecular weight of about 83,000 Da and two sugar chains. Each molecule of lactoferrin binds 2 atoms of Fe^{3+} in presence of sodium bicarbonate. The main function of this protein is to reduce the amount of free iron in biological liquids, inhibiting the bacterial growth (the bacteriostatic property of lactoferrin is already well known) and decreasing the risk of forming free radicals due to the presence of not chelated iron. Lactoferrin belongs to a family of glycoprotein generally called "Transferrin", characterized in so that they possess two binding sites for Fe^{3+} per molecule and a high degree of sequence homology among them.

Lactoferrin can be industrially produced by purification from bovine milk. We mainly used bovine lactoferrin in the experiments described in the present disclosure but the same antiviral activity is possessed by all the marketable proteins known as lactoferrin such as human lactoferrin and lactoferrin from mouse, produced by extraction or by recombinant DNA technique, and, therefore, the antiviral activity of lactoferrin has to be extended, as described in the present disclosure, to all the lactoferrins from various natural or synthetic sources.

EXAMPLES

Cell cultures

The cultured cell utilized are different according with the tested virus. For testing HSV1, VERO cells have been utilized while for testing *Rhinovirus* we utilized HeLa cells. Both cell lines have been cultured as monolayers at 37°C in Eagle's MEM containing 1.2 g/l $NaCO_3$, 2 mM glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 10% heat inactivated faetal calf serum in 5% CO_2 on 24- well tissue culture clusters (costar) by loading 0.5 ml of a suspension of $2x10^5$ HeLa cells/ml.

Unless otherwise indicated, lactoferrin and ovotransferrin used in the following examples are 30 % iron-saturated.

Example 1

Activity of lactoferrin and desferrioxamine methanesulfonate and related substances on the very early interactions between cell and viruses belonging to different genera.

The utilized procedure is the following:

a) incubation at 4°C for 1 h of the tested substances with cultured cells. After this period, the monolayer is washed and incubated at 37°C to allow viral infection;
b) incubation at 4°C for 1 h of the tested substances with the tested viruses. After this period, the monolayer is washed and incubated at 37°C to allow viral infection;
c) incubation at 4°C for 1 h of the tested substances with the virus and the cell monolayers. After this period, the monolayer is incubated at 37°C to allow viral infection.

The data concerning the activity of the lactoferrin (or other substances) are expressed as percentage of lysis plaques in comparison with the control in which the virus is incubated in its (or their) absence. The cultured cell utilized are different according with the tested virus. For testing HSV1, VERO cells have been utilized, while for testing *Rhinovirus* we utilized HeLa cells. Both cell lines have been cultured as monolayers at 37°C in Eagle's MEM containing 1.2 g/l $NaCO_3$, 2 mM glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 10% heat inactivated faetal calf serum in 5% CO_2 on 24- well tissue culture clusters (costar) by loading 0.5 ml of a suspension of $2x10^5$ HeLa cells/ml.

Lactoferrin and glycosilate ovotransferrin have been used at the concentration of 1 mg/ml; desferrioxamine and 8-idrossiquinoline at the concentrations of 0.03 mg/ml.

Table 2

Effect of transferrins and chelators on the replication of different virus.		
Conditions	Plaque forming units (%)	
	HSV1	RHINOVIRUS
Control	100	100
Bovine lactoferrin	100	100
Ovotransferrin	100	100
Glycosilate ovotransferrin	100	100
Desferrioxamine methanesulfonate	80	90
8-Hydroxyquinoline	75	80

From the reported data, is evident that lactoferrin and other transferrins do not affect the intracellular phase and the replication of the viruses.

A light effect is observed just for desferrioxamine methanesulfonate and 8-hydroxyquinoline for their well known mechanism of binding the intracellular iron necessary for the viral activity.

Other experiments have been carried out using the same concentration of bovine lactoferrin and ovotransferrin completely saturated with different metal ions. The data are reported in Table 3.

Table 3

Effect of lactoferrin saturated with different metal ions on the replication of different virus.		
Conditions	Plaque forming units (%)	
	HSV1	RHINOVIRUS
Control	100	100
Bovine lactoferrin in apo form	30	30
Bovine lactoferrin iron saturated	0	0
Bovine lactoferrin zinc saturated	40	40
Bovine lactoferrin cupper saturated	10	20
Bovine lactoferrin manganese saturated	0	0
Bovine lactoferrin cobalt saturated	50	30
Bovine lactoferrin nichel saturated	30	40

From the data reported in Table 3 it can be notice that lactoferrin also in saturated forms shows a noticeable antiviral activity.

Example 3

Activity of the transferrins-chelators association.

The maximum of the antiviral activity can be obtained when the substances (lactoferrin or giycosilate ovotransferrin and desfenrioxamine methanesulfonate or 8-hydroxyquinoline) exert all together their action i.e. the inhibition of the virus-cell interaction by lactoferrin or glycosilate ovotransferrin and the inhibition of replication by desferrioxamine methanesulfonate or 8-hydroxyquinoline.

Example 5 - Ointment

Composition per 100 g

Active ingredients:

Lactoferrin, from bovine milk, SIGMA Chemical Co., cat L4765, 10 g
Desferroxamine methanesulfonate, Ciba Geigy, 0.1 g

Carriers, preservatives agents:

Paraffin oil 10 g
Vaseline 80 g

Use

Apply on the skin twice a day

Example 6 - Cream

Composition per 100 g

Active ingredients:

Lactoferrin, from bovine milk, SIGMA Chemical Co., cat L4765, 10 g
Desferroxamine methanesulfonate, Ciba Geigy, 0.1 g

Carriers, preservatives agents:

Paraffin oil 6 g
Chlorocresol 0.1 g
Vaseline 15 g
Ketostearil alcohol 7.2 g
Polyethylene glycol monocethyl ether 1,8 g
Distilled water up to 100 g

Use

Apply on the skin twice a day.

Example 7 - Gel

Composition per 100 g

Active ingredients:

Lactoferrin, from bovine milk, SIGMA Chemical Co., cat L4765, 10 g
Desferroxamine methanesulfonate, Ciba Geigy, 0.1 g

Carriers, preservatives agents:

Carriers, preservatives agents:

Methyl-p-hydroxybenzoate	100 mg
Propyl-p-hydroxybenzoate	20 mg
Sodium phosphate monobasic	200 mg
Sodium EDTA	50 mg
Distilled water up to	100 g

Use

Spray into the nostrils twice a day

Example 10 - Nasal drops

Composition per 100 ml

Active ingredients:

Lactoferrin, from bovine milk, SIGMA Chemical Co., cat L4765, 10 g
Desferroxamine methanesulfonate, Ciba Geigy, 0.1 g

Carriers, preservatives agents:

Methyl-p-hydroxybenzoate	100 mg
Propyl-p-hydroxibenzoate	20 mg
Sodium chloride	0.8 g
Distilled water up to	100 ml

Use

Drop 0.5 mls of solution per each nostril twice a day

Claims

1. Preparation for preventing and curing acute and recurrent viral infetious diseases concerning skin, mucosas and other human or animal tissues characterized in that it comprising at least one transferrin and at least one molecule selected from the groups of chelators of iron and other metal ions.

2. Preparation according to claim 1, wherein the transferrin, in apo or iron or other metal ions saturated forms, at a protein concentration of 5 to 99% is represented by the bovine or human lactoferrin or by other natural or chemically modified transferrin characterized by high sequence homology with the protein known as lactoferrin, for example glycosilate ovotransferrin.

3. Preparation according to claim 1, wherein the chelator of iron and other metal ions at a concentration of 1 to 95%

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(54) **Preparation of lactoferrin (or analogous proteins) and desferrioxamine methanesulfonate (or other metal ion chelators) for the therapy of viral infectious diseases**

(57) The present invention relates to the therapeutic utilizzation of the preparation of lactoferrin and desferrioxamine metalsulphonate for the therapy of many acute or recurrent viral infectious diseases in humans and animals. In details, the present invention demonstrates the antiviral activity, based on the inhibition either of the absorption either of the replication of several virus, possessed by a preparation of lactoferrin (or its anologous proteins like transferrins) in apo or iron or other metal ions saturated forms, together with desferrioxamine metansulfonate (or other metal ion chelators like 8-idroxiquinoline 1,10-phenanthroline, phosphonoacetic acid). This antiviral activity is well evident towards DNA virus; like *Herpesviruses*, and towards RNA virus, like *Rhinovirus*, and can be generally extended and utilized for the therapy of many acute or recurrent viral infections concerning skin, mucosas or other tissues.

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ATTACHMENT A

Merriam-Webster's Collegiate® Dictionary

ELEVENTH EDITION

Merriam-Webster, Incorporated
Springfield, Massachusetts, U.S.A.

ture) c : CHANCE 4a ⟨a ~ at winning the prize⟩ d : a single appearance as an entertainer ⟨did a guest ~ for the program⟩ 7 : an effective remark; *esp* : SWIPE 2 ⟨a parting ~⟩ 8 a : a single photographic exposure; *esp* : SNAPSHOT b : a single sequence of a motion picture or a television program shot by one camera without interruption 9 : a charge of explosives 10 a : a small measure or serving (as one ounce) of undiluted liquor or other beverage ⟨vodka ~*s*⟩ ⟨a ~ of espresso⟩ b : a small amount applied at one time : DOSE ⟨a ~ of fertilizer⟩ ⟨a ~ of humor⟩ 11 **shot** *pl* : SPRINKLES, JIMMIES — **a shot** : for each one — **like a shot** : very rapidly — **shot in the arm** : STIMULUS, BOOST — **shot in the dark** 1 : a wild guess 2 : an attempt that has little chance of success

[2]**shot** *past and past part of* SHOOT

[3]**shot** *adj* (1763) 1 a *of a fabric* : having contrasting and changeable color effects : IRIDESCENT b : suffused or streaked with a color ⟨hair ~ with gray⟩ c : infused or permeated with a quality or element ⟨~ through with wit⟩ 2 : having the form of pellets resembling shot 3 : reduced to a ruined or useless state ⟨his nerves are ~⟩

shot clock *n* (1973) : a clock in basketball that displays a countdown of the time within which shooting the ball is required

[1]**shot·gun** \ˈshät-ˌgən\ *n* (1776) 1 : a smoothbore shoulder weapon for firing shot at short ranges 2 : an offensive football formation in which the quarterback plays a few yards behind the line of scrimmage and the other backs are scattered as flankers or slotbacks — **shotgun** *vt* — **shot·gun·ner** \-ˌgə-nər\ *n*

[2]**shotgun** *adj* (1892) 1 : of, relating to, or using a shotgun 2 : involving coercion 3 : covering a wide field with hit-or-miss effectiveness

shotgun house *n* (1940) : a house in which all the rooms are in direct line with each other usu. front to back — called also *shotgun cottage, shotgun shack*; compare RAILROAD FLAT

shotgun marriage *n* (1929) 1 : a marriage forced or required because of pregnancy — called also *shotgun wedding* 2 : a forced union ⟨a spate of brokerage mergers . . . hastily arranged *shotgun marriages* —John Brooks⟩

shot hole *n* (1875) 1 : a drilled hole in which a charge of dynamite is exploded 2 : the dropping out of small rounded fragments of leaves that produces a shot-riddled appearance and is caused esp. by parasitic action

shot put *n* (1898) : a field event in which a shot is heaved for distance — **shot–put·ter** \ˈshät-ˌpu̇-tər\ *n*

shot·ten \ˈshä-tᵊn\ *adj* [ME *shotyn*, fr. pp. of *sheten* to shoot] (15c) : having ejected the spawn and so of inferior food value ⟨~ herring⟩

should \shəd, ˈshu̇d\ *verbal auxiliary, past of* SHALL [ME *sholde*, fr. OE *sceolde* owed, was obliged to, ought to] (bef. 12c) 1 — used in auxiliary function to express condition ⟨if he ~ leave his father, his father would die —Gen 44:22(RSV)⟩ 2 — used in auxiliary function to express obligation, propriety, or expediency ⟨'tis commanded I ~ do so —Shak.⟩ ⟨this is as it ~ be —H. L. Savage⟩ ⟨you ~ brush your teeth after each meal⟩ 3 — used in auxiliary function to express futurity from a point of view in the past ⟨realized that she ~ have to do most of her farm work before sunrise —Ellen Glasgow⟩ 4 — used in auxiliary function to express what is probable or expected ⟨with an early start, they ~ be here by noon⟩ 5 — used in auxiliary function to express a request in a polite manner or to soften direct statement ⟨I ~ suggest that a guide . . . is the first essential —L. D. Reddick⟩

[1]**shoul·der** \ˈshōl-dər\ *n* [ME *sholder*, fr. OE *sculdor*; akin to OHG *scultra* shoulder] (bef. 12c) 1 a : the laterally projecting part of the human body formed of the bones and joints with their covering tissue by which the arm is connected with the trunk b : the region of the body of nonhuman vertebrates that corresponds to the shoulder but is less projecting 2 a : the two shoulders and the upper part of the back — usu. used in pl. b *pl* : capacity for bearing a task or blame ⟨placed the guilt squarely on his ~*s*⟩ 3 : a cut of meat including the upper joint of the foreleg and adjacent parts — see LAMB illustration 4 : the part of a garment at the wearer's shoulder 5 : an area adjacent to or along the edge of a higher, more prominent, or more important part: as a (1) : the part of a hill or mountain near the top (2) : a lateral protrusion or extension of a hill or mountain b : either edge of a roadway; *specif* : the part of a roadway outside of the traveled way 6 : a rounded or sloping part (as of a stringed instrument or a bottle) where the neck joins the body — **shoul·dered** \-dərd\ *adj*

[2]**shoulder** *vb* **shoul·dered; shoul·der·ing** \-d(ə-)riŋ\ *vt* (14c) 1 : to push or thrust with or as if with the shoulder : JOSTLE ⟨~*ed* his way through the crowd⟩ 2 a : to place or bear on the shoulder ⟨~*ed* her knapsack⟩ b : to assume the burden or responsibility of ⟨~ the blame⟩ ~ *vi* : to push with or as if with the shoulders aggressively

shoulder bag *n* (1912) : a handbag looped over the shoulder by a strap

shoulder belt *n* (1967) : an automobile safety belt worn across the torso and over the shoulder — called also *shoulder harness*

shoulder blade *n* (14c) : SCAPULA

shoulder board *n* (1942) : one of a pair of broad pieces of stiffened cloth worn on the shoulders of a military uniform and carrying insignia

shoulder girdle *n* (1868) : the bony or cartilaginous arch that supports the forelimbs of a vertebrate — called also *pectoral girdle*

shoulder knot *n* (1676) 1 : an ornamental knot of ribbon or lace worn on the shoulder in the 17th and 18th centuries 2 : a detachable ornament of braided wire cord worn on ceremonial occasions on the shoulders of a uniform by a commissioned officer

shoulder patch *n* (1945) : a cloth patch bearing an identifying mark and worn on one sleeve of a uniform below the shoulder

shoulder strap *n* (1688) : a strap that passes across the shoulder and holds up an article or garment

should·est \ˈshu̇-dəst\ *archaic past 2d sing of* SHALL

shouldn't \ˈshu̇-dᵊnt, -dᵊn, *dial also* ˈshu̇-tᵊn(t) *or* ˈshu̇nt\ (1675) : should not

shouldst \shədst, ˈshu̇dst, shətst, ˈshu̇tst\ *archaic past 2d sing of* SHALL

[1]**shout** \ˈshau̇t\ *vb* [ME] *vi* (14c) 1 : to utter a sudden loud cry 2 : to command attention as if by shouting ⟨a quality that ~*s* from good novels —John Gardner⟩ ~ *vt* 1 : to utter in a loud voice 2 : to cause to be, come, or stop by or as if by shouting ⟨~*ed* himself hoarse⟩ ⟨the proponents ~*ed* down the opposition⟩ — **shout·er** *n*

[2]**shout** *n* (14c) : a loud cry or call

shouting distance *n* (1930) : a short distance : easy reach — usu. used with *within* ⟨lived within *shouting distance* of her cousins⟩

shout–out \ˈshau̇t-ˌau̇t\ *n* (1990) : a brief expression of greeting or praise given esp. on a broadcast or audio recording

shout song *n* (1925) : a rhythmic song sung at religious services esp. by black Americans and characterized by responsive singing or shouting between leader and congregation

[1]**shove** \ˈshəv\ *vb* **shoved; shov·ing** [ME, fr. OE *scūfan* to thrust away; akin to OHG *scioban* to push and prob. to Lith *skubti* to hurry] *vt* (bef. 12c) 1 : to push along 2 : to push or put in a rough, careless, or hasty manner : THRUST 3 : to force by other than physical means : COMPEL ⟨~ a bill through the legislature⟩ ~ *vi* 1 : to move by forcing a way ⟨bargain hunters *shoving* up to the counter⟩ 2 a : to move something by exerting force b : LEAVE — usu. used with *off* ⟨*shoved* off for home⟩ — **shov·er** *n*

[2]**shove** *n* (14c) : an act or instance of shoving : a forcible push

[1]**shov·el** \ˈshə-vəl\ *n* [ME, fr. OE *scofl*; akin to OHG *scūfla* shovel, OE *scūfan* to thrust away] (bef. 12c) 1 a : a hand implement consisting of a broad scoop or a more or less hollowed out blade with a handle used to lift and throw material b : something that resembles a shovel c : an excavating machine; *esp* : a hydraulic diesel-engine driven power shovel 2 : SHOVELFUL

[2]**shovel** *vb* **-eled** *or* **-elled; -el·ing** *or* **-el·ling** \ˈshə-və-liŋ, ˈshəv-liŋ\ *vt* (15c) 1 : to take up and throw with a shovel 2 : to dig or clean out with a shovel 3 : to throw or convey roughly or in a mass as if with a shovel ⟨~*ed* his food into his mouth⟩ ~ *vi* : to use a shovel

shov·el·er *or* **shov·el·ler** \ˈshə-və-lər, ˈshəv-lər\ *n* (15c) 1 : one that shovels 2 : any of several freshwater ducks (genus *Anas*) that have a large very broad bill and feed by dabbling

shov·el·ful \ˈshə-vəl-ˌfu̇l\ *n, pl* **shovelfuls** \-ˌfu̇lz\ *also* **shov·els·ful** \-vəlz-ˌfu̇l\ (1533) : as much as a shovel will hold

shovel hat *n* (1829) : a shallow-crowned hat with a wide brim curved up at the sides that is worn by some clergymen

shov·el·nose \-ˌnōz\ *n* (1709) : a shovel-nosed animal and esp. a fish

shov·el–nosed \ˈshə-vəl-ˌnōzd\ *adj* (1707) : having a broad flat head, nose, or beak

shovel pass *n* (1940) : a short underhand pass (as in football)

[1]**show** \ˈshō\ *vb* **showed** \ˈshōd\; **shown** \ˈshōn\ *or* **showed; show·ing** [ME *shewen, showen*, fr. OE *scēawian* to look, look at, see; akin to OHG *scouwōn* to look, look at, and prob. to L *cavēre* to be on one's guard] *vt* (12c) 1 : to cause or permit to be seen : EXHIBIT ⟨~*ed* pictures of the baby⟩ 2 : to offer for sale ⟨stores were ~*ing* new spring suits⟩ 3 : to present as a public spectacle : PERFORM 4 : to reveal by one's condition, nature, or behavior ⟨~*ed* themselves to be cowards⟩ 5 : to give indication or record of ⟨an anemometer ~*s* wind speed⟩ 6 a : to point out : direct attention to ⟨~*ed* the view from the terrace⟩ b : CONDUCT, USHER ⟨~*ed* me to an aisle seat⟩ 7 : ACCORD, BESTOW ⟨~*s* them no mercy⟩ 8 a : to set forth : DECLARE b : ALLEGE, PLEAD — used esp. in law ⟨~ cause⟩ 9 a : to demonstrate or establish by argument or reasoning ⟨~ a plan to be faulty⟩ b : INFORM, INSTRUCT ⟨~*ed* me how to solve the problem⟩ 10 : to present (an animal) for judging in a show ~ *vi* 1 a : to be or come in view ⟨3:15 ~*ed* on the clock⟩ b : to put in an appearance ⟨failed to ~⟩ 2 a : to appear in a particular way ⟨anger ~*ed* in their faces⟩ b : SEEM, APPEAR 3 a : to give a theatrical performance b : to be staged or presented 4 a : to appear as a contestant b : to present an animal in a show 5 : to finish third or at least third (as in a horse race) 6 : to exhibit one's artistic work — **show·able** \ˈshō-ə-bəl\ *adj* — **show one's hand** *also* **show one's cards** 1 : to display one's cards faceup 2 : to declare one's intentions or reveal one's resources — **show one the door** : to tell someone to get out; *also* : FIRE 2b

syn SHOW, EXHIBIT, DISPLAY, EXPOSE, PARADE, FLAUNT mean to present so as to invite notice or attention. SHOW implies no more than enabling another to see or examine ⟨*showed* her snapshots to the whole group⟩. EXHIBIT stresses putting forward prominently or openly ⟨*exhibit* paintings at a gallery⟩. DISPLAY emphasizes putting in a position where others may see to advantage ⟨*display* sale items⟩. EXPOSE suggests bringing forth from concealment and displaying ⟨sought to *expose* the hypocrisy of the town fathers⟩. PARADE implies an ostentatious or arrogant displaying ⟨*parading* their piety for all to see⟩. FLAUNT suggests a shameless, boastful, often offensive parading ⟨nouveaux riches *flaunting* their wealth⟩.

syn SHOW, MANIFEST, EVIDENCE, EVINCE, DEMONSTRATE mean to reveal outwardly or make apparent. SHOW is the general term but sometimes implies that what is revealed must be gained by inference from acts, looks, or words ⟨careful not to *show* his true feelings⟩. MANIFEST implies a plainer, more immediate revelation ⟨*manifested* musical ability at an early age⟩. EVIDENCE suggests serving as proof of the actuality or existence of something ⟨a commitment *evidenced* by years of loyal service⟩. EVINCE implies a showing by outward marks or signs ⟨*evinced* not the slightest fear⟩. DEMONSTRATE implies showing by action or by display of feeling ⟨*demonstrated* their approval by loud applause⟩.

[2]**show** *n, often attrib* (13c) 1 : a demonstrative display ⟨a ~ of strength⟩ 2 a *archaic* : outward appearance b : a false semblance : PRETENSE ⟨made a ~ of friendship⟩ c : a more or less true appearance of something : SIGN d : an impressive display ⟨his role as househusband . . . was purely for ~ —John Lahr⟩ e : OSTENTATION 3 : CHANCE 2 ⟨gave him a ~ in spite of his background⟩ 4 : something exhibited esp. for wonder or ridicule : SPECTACLE 5 a : a large display or exhibition arranged to arouse interest or stimulate sales ⟨the national auto ~⟩ b : a competitive exhibition of animals (as dogs) to demonstrate quality in breeding 6 a : a theatrical presentation b : a radio or television program c : ENTERTAINMENT 3b(1) 7 : ENTERPRISE, AFFAIR ⟨they ran the whole ~⟩ 8 : third place at the finish (as of a horse race) 9 *often cap* : the major leagues in baseball — used with *the*

\ə\ abut \ᵊ\ kitten, F table \ər\ further \a\ ash \ā\ ace \ä\ mop, mar \au̇\ out \ch\ chin \e\ bet \ē\ easy \g\ go \i\ hit \ī\ ice \j\ job \ŋ\ sing \ō\ go \ȯ\ law \ȯi\ boy \th\ thin \t͟h\ the \ü\ loot \u̇\ foot \y\ yet \zh\ vision, beige \k, ⁿ, œ, ue, ʸ\ see Guide to Pronunciation

ATTACHMENT B

Third Enlarged Edition

CONCISE CHEMICAL and TECHNICAL DICTIONARY

Edited by
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er formed on iron and steel during fabrication.
millstone. See buhrstone.
"Milltrox." Wet-milled zircon.
mill, universal. Two-high or three-high rolling mill with both horizontal and vertical rolls; frequently, the vertical rolls are doubled, with one pair on each side of the horizontal rolls.
Milmer. Copper δ -quinolinolate; used as textile preservative.
"Milogard." propazine.
Milontin. phensuximide.
Milorganite. See nitrorganic.
milori blue. Prussian blue with slight bronze overtones; besides the ferric ferrocyanide, it may contain gypsum, alum, barium sulfate or chalk; used in the manufacture of laundry blue, lithographic and printing inks, paints, coloring soaps, fertilizer mixtures.
milori green. Yellowish green pigment, chiefly lead chromate.
"Miltown." mepropamate.
Milvex. nylon film.
Milvex. nylon resin.
Milwaloy. Corrosion-resistant, high-alloy steels, with up to 30% chromium and up to 15% nickel.
mimetite (memetesite). $3Pb_3As_2O_8.PbCl_2$; yel., br., wh. or col. hex. min.; sp.gr. 6.98-7.25; hardness 3.5.
mimosine. 3-hydroxy-4-oxo-1 (4H) pyridinealanine.
min. Minute; minium (Apothecary); minimum; mineral.
min (subscript). Minimum.
minargent. Cooper-base alloys, high in nickel (32 to 40% with fractional percentages of aluminum and other elements; used as a silver substitute.
mineral. Solid inorganic element or compound occurring naturally in the earth's crust, *e.g.* native copper, bauxite, etc.
mineral black. Carbonaceous rock or mineral powder; used as a black pigment.
mineral blue. See iron ferrocyanide (ic).
mineral brown. Metallic brown (see)
mineral butter. See antimony trichloride.
Mineral Colloid --. montmorillonite.
mineral colza oil. Mineral burning oil.
mineral cotton. See slag wool and rock wool.
mineral dressing. Processing or raw minerals to yield marketable products and waste by means that do not destroy the physical and chemical identity of the minerals.
Mineralead. Sulfur and silica.
mineral, economic. Mineral of commercial value.
mineral fat. See petrolatum.
mineral green. See mountain green.
Mineralites Powdered mica.
mineralizer. Small quantity of flus, *e.g.*, lime, added to a ceramic batch to promote crystal growth or compound formation.
mineral jelly, See petrolatum.
mineralogical hardness. See Moh's hardness.
mineralogy. Study of rocks and minerals.
mineral oil (white liquid paraffin; liquid petrolatum; Alboline; adepsine oil; Paroleine; Saxol). Mixture of liquid hydrocarbons obtained from petroleum; col. trans. liq.; sp.gr. 0.828-0.905[25]; b.p. 360; i.w.; s.eth.; s.bz.; used in paints, varnishes, lacquers, medicine, sprays, as a solvent.
mineral orange. See red lead.
mineral red. See red lead.
mineral rouge. See red iron oxide.
mineral rubber (gilsonite; elaterite). Fossil resin resembling asphaltum, found only in U.S.; dk. br. sol.; sp.gr. 1.065-1.070; m.p. 160-170; used in varnishes, protective coatings, in insulating, paving and waterproofing.
mineral seal oil. Distillate with a boiling range between those of kerosene and gas oil proper; used for illuminating lighthouses.
mineral spirits. Petroleum solvent; b.p. 153.3-203.9.
mineral spirits #10. Petroleum solvent; b.p. 152.2-197.8.
mineral tallow. See bitumen.
mineral tar. Soft natural asphalt.
mineral wax. See ozokerite wax.
mineral white. barium sulfate.
mineral wool (slag wool). Fibrous material, resulting from the action of a jet of steam on molten slag; used in insulating.
mineral yellow. yellow ocher.
"Minerec." Dixanthogen.
Minerol. solubilized light mineral oil.
miners' inch. In mining, aperture, one inch square with the upper edge 6 inches below the surface of a stream, which is used to measure rate of flow; yield about 1.5 cubic feet per minute.
miners' oil. Mineral burning oil mixed with refined white fish oil.
"Minicel." polypropylene foam sheet.
Minicel L-200. Cross-linked polyethylene foam.
"Minicel" S-2000. Cross-linked polyethylene foam.
"Minifos." monoammonium phosphate.
minimum deviation. Deviation or change of direction of light passing through a prism when the angle of incidence equals the angle of emergence.

mini-plant. Small plant
chemical processes, inte
between laboratory and
minium. See red lead.
Minkowski's theorem. E
place in a four-dimensi
continuum.
Minofor. Tin-base alloy
mately 19% antimony,
copper, and optionally
Minol. Explosive, used
charges, consisting of T
nitrate and aluminum p
Minoline Yellow mixtur
castor, mineral and o
used as a softening a
cotton and wool.
mint. See spearmint.
mint camphor. Menthol.
"Mintezol." thiabendazol
Mintrex. solvent naphtha
Mintrol. solvent naphtha
Min-U-Gel. attapulgite.
minus-blue. Substance th
cipally blue light.
minus-green. Substance
principally green light.
Min-U-Sil. Micron sized
minus mesh. Portion of
ple passing through a gi
minus-red. Substance tha
cipally red light.
Minvar. Cast ferrous al
38% nickel and 1 to 3%
expansion alloy.
"Miochol." acetylcholine
mannitol.
Miokon. diprotrizoate.
"Mipafox." *bis*(monoi
fluorophosphineoxide.
Mi-Phos M-5. mangan
precipitating compound.
Mi-Phos Z-3. zinc phosp
ing compound.
"Mi-Phos" 6. iron phosp
Mi-Phos 8. iron phospha
compound.
Mipolam. Polyvinyl ch
non-inflammable, rubb
electrical insulation.
Mipol M-20. Rubber-lik
microcrystalline wax in r
"Mir." lauryl sulfate.
mirabilite (Glauber salt).
wh. monocl. min.; sp.
hardness 1.5-2.0.
Miracil. 1-Diethylamin
methylthioxanthone; us
Mira-Creme. starch.
Miradon. anisindione.
"Miragene" T. fatty acid
Miralite. Alloy with 96%

casein of the milk.
paracetomol. See *p*-hydroxy acetanilide.
parachloramine [1-(*p*-chloro-α-phenylbenzyl)-4-(m-methylbenzyl)piperazine meclizine; meclozine]. $C_{25}H_{27}ClN_2.2HCl$; m.w. 465; cr.; i.w.; s.al.; s.chl.
parachor. True measure of molecular volume as determined from Sugden's function which accounts for the surface tension.
Paracide. *p*-dichlorbenzol.
"Paricin" 9. propyleneglycol monohydroxy-stearate.
Paracin I. Methyl hydroxy stearate.
paracodin tartrate (dihydrocodeine bitartrate). $C_{18}H_{23}NO_2.C_4H_6O_6.H_2O$; m.w. 469.25; wh. cr.; d. 187; s.w.; s.al.
"Paracol." wax and wax-rosin emulsions.
Paracon. High molecular weight condensation product of hydroxy acids or of dibasic acids with glycols.
paraconic acid (tetrahydro-5-oxo-3-furan carboxylic acid; itamalic acid γ-lactone). $CH_2COOCH_2CHCOOH$; m.w. 130.05; cr., deliq.; m.p. 58; s.w.
"Paracortol." preonisolone.
paracoto (coto). Dried bark of a Bolivian tree containing paracotoin, hydrocotoin, leucotin.
paracotoin. $C_{12}H_8O$; m.w. 216.06; lt.-yel. cr.; m.p. 162; s.al.; s.eth.
paracoumarone. See coumarone-indene resin.
"Paracril." nitrile rubber.
paracrystalline. Term applied to grouping of molecules just prior to crystallization.
paracumarone. See coumarone-indene resin.
paracyanogen. (CN)x; m.w. (26.01)x; br. powd.; i.w.
paradamite. Zn_2AsO_4OH. min.
Paradene. Paracumarone-indene resin.
Paradione. paramethadione.
paradise plant. See mezereum.
paradise-seed oil (grains of paradise oil). Essential oil distilled from seeds of Amomum melegueta; odor similar to that of cardamon oil.
paradol (gingerol). $C_9H_{14}O_2$; m.w. 154.11; yel. semi-liq.; sp.gr. 1.069; i.w.; s.al.; s.eth.; amaroid.
Paradol. Condensation product of methylene and cresolsulfonic acid.
Paradone. Vat dye.
Paradow. *p*-dichlorobenzene.
Paradura. Phenolic resin.
"Paradyne." dipyrone.
paraffin-base oil. Petroleum which yields a residue of solid paraffin on distillation.
paraffin, chlorinated. See chlorinated paraffin.
paraffinicity. Proportion of paraffins, in a petroleum, relative to naphthenes and other compounds.
Paraffin Inhibitor Sticks. micro-crystalline wax and amorphous polyethylene.
paraffin jelly. See petrolatum.
parraffin liquid. mineral oil.
parraffin native. See ozokerite wax.
paraffin,neo. See neoparaffin.
paraffin oil. See mineral oil.
paraffins. See alkanes.
paraffin scale. Crude paraffin wax.
paraffin series. Hydrocarbons of formula C_nH_{2n+2}.
paraffin slack wax. See slack wax.
paraffin wax (paraffin; hard paraffin). Solid mixture of puried petroleum hydrocarbons; odorl. col. or wh. mass, sp.gr. ca. 0.9; m.p. 50-57; i.w.; s.eth.; s.bz.
Paraffion. Paraffin wax emulsion containing aluminum sulfate.
Parafilm. Paraffin wax modified with an elastomer to give a flexible, thermoplastic sheet or film.
"Paraflex." chlorzoxazone.
Paraflint. F-T wax.
Paraflow. Viscous oil made by condensation of chlorinated wax with an aromatic hydrocarbon.
paraformaldehyde. See polyoxy methylene.
Paragerm. Methyl-propyl-diphenol *p*-oxybenzoate.
Paraglas. polyacrylate.
Paragon clay. Hydrous aluminum silicate.
"Paragon" TS. alkyl naphthalene sulfonate + triethanolamine and alkali.
Paraguay tea. See yerba maté.
"Paragum" #6. sodium polyacrylate.
"Paragum" 60. ammonium salt of a polycarboxylic acid copolymer.
Paragutta. Deproteinized rubber, gutta percha hydrocarbon, and wax.
parahematin. Compound of hematin with denatured proteins, nitrogenous bases and the iron atom in the ferric state.
para-hydrogen. Hydrogen molecules with anti-parallel nuclear spins and even rotational quantum numbers.
paraison. Bubble (glass) formed at end of blowers tube.
"Paral." paraldehyde.
Paralac. alkyd resin.
Paralaudin. Diacetyl-dihydro morphine.
paraldehyde (2,4,6-trimethyl 1,3,5-trioxane; paraacetaldehyde). $OCH(CH_3)OCH(CH_3)OCHCH_3$ m.w.

ATTACHMENT C

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Pestox 14. See dimefox.
PET. poly(ethylene terephalate).
pet. Petroleum.
petalite. $Li_2O.Al_2O_3.8SiO_2$; col., wh., gray, redsh. or grnsh. monocl. min.; sp.gr. 2.386-2.465; hardness 6.0-6.5.
petaloid. Resembling a flower petal.
petit-grain oil (petit-grain citronier oil). Oil distilled from bitter-orange tree leaves and unripe fruit; yel.; sp.gr. 0.887-0.900$^{15/15}$; sl.s.w.; s.al.
PETN. See Penthrite.
Petralol. Liquid petrolatum.
Petramin. chelating dye.
Petrex 5. Glycol ester of terpene-maleic anhydride addition product.
Petrex acid. reaction product of maleic anhydride and a monocyclic terpene.
Petrex resins. Alkyd resins produced from Petrex acid.
"Petrex" SS-70A. terpene alkyd resin.
Petri-dish. Shallow, circular dish with a loosely fitting, overlapping cover; used for culturing microorganisms on a solid medium.
petrified wood (agatized wood; opalized wood; silicified wood). Wood-like mineral consisting of opal or agate.
petri-, petro-. Prefix meaning stone.
Petro. Liquid petrolatum.
"Petro" AA (Surfatrope). methyl naphthalene sodium sulfonate.
Petro BA, BAF, BP, WP, SWP, (11, 208). sodium xylene sulfonates.
"Petro" 250. Complex dimethyl naphthalene sulfonate.
Petro 348. linear alkyl sulfonates.
Petroacid. Mixture of fatty acids formed from oxidation of petroleum fractions.
"Petrobase" No. 2. petroleum sulfonate and emulsifier.
Petrobenzol. Petroleum solvent; boiling range 61.1-96.1
Petrocera. beeswax (335) and petrolatum (665).
petrochemicals. Chemicals present in or derived from natural gas or crude petroleum by physical refining or chemical reaction.
Petroflex. butadiene-styrene elastomer.
Petrohol. isopropyl alcohol.
Petroil. petrolatum.
petrol. See gasoline.
petrolatum (petroleum jelly; mineral jelly; Vaseline; paraffin jelly; cosmoline; mineral fat; petroleum ointment; saxoline). Purified mixture of semisolid hydrocarbons; lt. yel. to amber amor.; sp.gr. 0.820-0.850; m.p. 45-48; i.w.; s.eth.; s.bz.
petrolatum, liquid. white mineral oil.
petrolatum, white. See Albolene.
Petrolene. Petroleum solvent; boiling range 143-202°F.
petrolenes (malthenes). Portions of bituminous substances soluble in hexane.
petroleum (rock oil; coal oil; Seneca oil; mineral oil; crude oil; earth oil; Lima oil). Hydrocarbon mixture obtained from the earth; sp.gr. 0.78-0.97; yel.-black bisc. liq., inflam.; i.w.; s.eth.
petroleum acids, green. Water-soluble acids from acid petroleum sludge.
petroleum benzin. See benzin.
petroleum benzin. Petroleum ether (see).
petroleum, blown. Native liquid bitumen heated and oxidized by blown air.
petroleum, coastal type. Asphalt-base petroleum, naphthenic in character, containing little or no paraffin wax.
petroleum, core. Petroleum plus core gas in core as brought to surface in drilling wells.
petroleum ether (canadol; light ligroin; benzine). Fraction of petroleum distilling between 40 and 70°; col. liq., inflam.; sp.gr. 0.635-0.660; s.al.; s.eth.; consists of pentanes and hexanes.
petroleum jelly. petrolatum.
petroleum, mid-continent. Mixed-base petroleum containing both paraffin wax and asphalt.
petroleum, mixed base. Petroleum containing paraffin hydrocarbons, asphaltic material and paraffin wax.
petroleum mobility. Ratio of effective permeability to the viscosity of hydrocarbons in place.
petroleum naphtha. Petroleum fraction distilling between 90 and 120°; sp.gr. 0.707-0.722; consists mainly of heptanes and octanes.
petroleum ointment. petrolatum.
petroleum, Pennsylvania type. Paraffin-base petroleum, containing paraffin wax but little or no asphalt.
petroleum pour point. The temperature 5°F. above that at which a petroleum oil will not flow when chilled without disturbance at specified cooling rate.
petroleum pour point, maximum. A figure for temperature, 5°F. above the highest temperature at which an oil ceases to flow when alternately cooled and warmed under definitely prescribed conditions.
petroleum, reduced crude. Residue from distillation of petroleum.
petroleum reservoir. A natural structural trap containing an exploitable concentration of hydrocarbons.
petroleum solvent. Solvent obtained from petroleum distillates, *e.g.*, petroleum ether, mineral spitits.

petroleum spirits.
petroleum spirits
fined petroleum
point not below
petroleum sulfona
fonate.
petroleum sulfonic
acid). Comple
acids of aromat
paraffins and
paraffins, obtai
finery sludge.
petroleum thinner
leum solvent.
petroleum, topped
additional volati
removed.
petrologen. kero
Petromor. petrol
Petronate. Sodiu
nates.
Petropol 2138.
olefinic hydroc
unsaturated vi
some drying-oil
Petropon. Synth
petroselaidic acid
acid.
Petrosene. Micr
Petroset. Solprer
"Petroset" SB.
"Solprene GEO
Petrosol C-50 (Pe
C-90). Petrole
Petrosol 25. Petr
fatty ester.
"Petro" Solve. a
vent.
"Petrosul" 745. s
+ min. oil.
"Petrothene." pol
"Petrothene" CD-
black.
"Petrothene" XL.
ethylene comp.
Petrothene XL141
ethylene.
Petro ULF-X. al
ium salt.
"Petrowet" R. so
hydrocarbon sul
"Petrowet" WN.
pettymorrel. See
PE tube. Photoel
petunin (methyl
glucoside from
brida.
pentunin chloride.
vlt. pl.; m.p. ca.
petzite. (Au.Ag)2
min.; sp.gr. 8.7
naturally occurri

amine.
Varonic T410. Ethoxylated tallow diamine.
Varonic Q110. Oleyl alcohol ethoxylate.
Varonic 3200. Castor oil ethoxylate.
Varonol ALS. lauryl alcohol sulfate, ammonium salt.
Varonol DLS. lauryl alcohol sulfate, diethanolamine salt.
Varonol MLS. lauryl alcohol sulfate, magnesium salt.
Varonol SLES. ethoxylated lauryl alcohol sulfate, sodium salt.
Varonol SLS. lauryl alcohol sulfate, sodium salt.
Varonol TAS. lauryl alcohol sulfate, triethanolamine salt.
Varox. 2,5-*bis*(*t*-butyl-peroxy)2-,5-dimethyl-hexane.
Varox 743. A 50% active coco dihydroxyethyl amine oxide.
Varox 1770. coco amine oxide.
Varsol. aliphatic petroleum solvent; b.p. 148.9-210.
Varsol 1. petroleum solvent.
Varsoy. Prebodied chemically reacted soybean oil.
"Varstat" K22. Ethoxylated coco alkanolamide.
Varstat T22. ethoxylated soya alkanolamide.
Varsulf 60. Biodegradable linear dodecyl benzene sulfonate as triethanolamine salt (60% solution).
Varsulf 65. Hard dodecyl benzene sulfonate, triethanolamine salt.
"Varsulf" 91. sodium octyl sulfate (40%) aq. sol'n.
vasaca. See adhatoda.
Vascoloy-Ramet. Tungsten and tantalum cemented carbides with nickel or cobalt as binding matrix.
Vasco steel. Carbon and low-alloy chromium-vanadium tool steels; alloy steels contain 0.15 to 0.25% vanadium, up to 1.5% chromium.
vasculose. Lignocellulose.
Vaseline. petroleum jelly.
vasicine. See *l*-peganine.
Vasiodone. diodone.
Vasite. Liquid stripping agent of the hydrosulfite type.
"Vasocon." maphazoline hydrochloride.
"Vasodilan." isoxsuprine HCl.
Vasogen. oxygenated petrolatum.
vasopressin. Hormone of the posterior lobe of the pituitary gland which causes contraction of the muscles of the arterioles and capillaries, with resulting rise in blood pressure, and which also causes contraction of the intestinal muscles.
Vasoxine. methoxamine HCl.
Vasoxyl hydrochloride. methoxamine hydrochloride.
Vasylox. methoxamine HCl.
Vatacid. Hydrogen leuco dispersion of a vat-dye.
vat dye. Dyestuff insoluble in water but soluble in alkaline solution when reduced with suitable agents, *e.g.*, the insoluble indigo blue is converted to soluble indigo white upon reduction. The latter is applied to the cloth and oxidized by exposure to air to the original insoluble blue form.
Vatrolite. See Lykopon.
Vatsol OS. sodium salt of alkyl naphthalene sulfonic acid.
Vatsol OT. dioctyl sodium sulfosuccinate.
Vatsol OT-C Dioctyl sodium sulfosuccinate and inert organic diluent.
Vattene. Vat dye.
vatting. Process of solubilizing vat dyes in an aqueous solution of caustic soda and sodium hydrosulfite.
vatting test. Re-oxidation and decolorization of reduced anthraquinones.
Vavilov's law. Constant quantum yield of fluorescence over broad ranges of wave-lengths.
Vaylar. Fine silica.
Vaylar 800. Arc-process silica.
"Vazo" Vinyl. alpha, alpha-azobisisobutyronitrile.
V-Bor. Borax pentahydrate.
VC. Vinylidene chloride plastic.
v-c-9-104. See prophos.
V-C-13. See nemacide.
VCA. vinyl chloride acetate.
Veba-Wax. New name for Ruhrwax.
Vectolite. Compressed powdered iron oxide and cobalt oxide.
vector. Straight line that, in both length and direction, represents a quantity, *e.g.*, velocity, stress.
vector field. Totality of values of a vector quantity which has a definite value at each point of a region.
vector function. Vector quantity having a definite value for every value within certain limits of an independent variable.
vector polygon. Force polygon for a set of forces whose resultant equals zero.
vector product. Vector having magnitude equal to product of magnitudes of the factors and sine of angle between the direction of the two vectors and a direction perpendicular to their common plane.
vector ratio. Ratio of two alternating quantities in which vectors express the relative amplitudes and phases.

Vee. polyvinylid
"Veegum." Moc aluminum silica
Veegum CER.
Veegum PRO.
veenite. 2PbS.(St
veering. Clockw tion of the winc
vees. Beveled ed
vee-way (V-way) machined in a c a moving part.
Vegadex. See CI
Vegadex. 2- carbamate.
"Vegamine." Hy tein.
Vegard-Kaplan ba caused by nitrog
Vegard law. Wh line materials fo lattice constant the space betwee constants in rati tities.
"Vegefat." Polyu
Vegemine. Hydro tein.
vegetable oxy-acid
vegetable rinse. C hair coloring.
Vegetex. Extracts
veg-gasoline. Mot cracking tung or
Vegifat. High tite
Vegol. Concentrat tocopherols; vita
Vegolysen. hexam
Vegolysen T. hex trate.
vehicle. Liquid p printing ink.
vehicle, paint. S paint, consisting drying oils, resins persing medium.
veiling. Cob-web
Vejin. Vegetable le
vel. Velocity.
Vel. Mixed cocon eryl sodium sulfor
Velan PF. pyridinium chloric
Velatrum. petroleu
Velban. Vinblastin
vellarin. Amaroid cotyle asiatica lea s.al.; s.eth.
vellosine. $C_{21}H_{22}O_2$ 396.23; yel. cr.; m s.eth.; toxic alkalo
velocity. Time rate direction.